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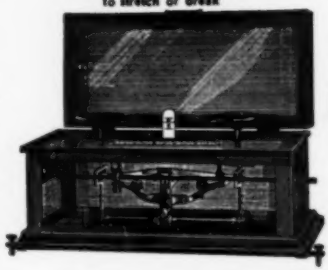

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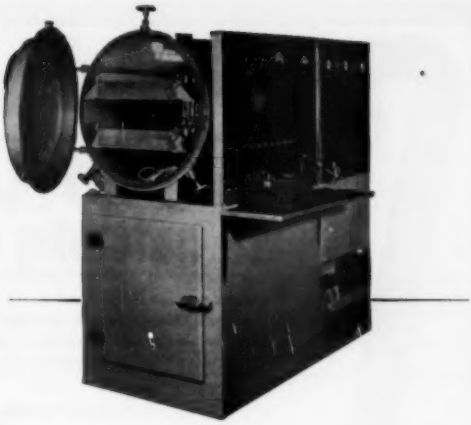

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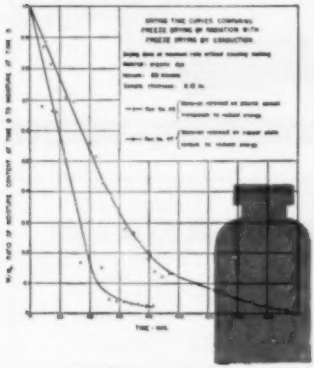

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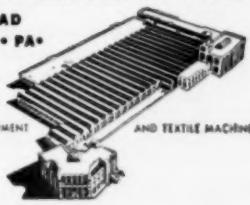

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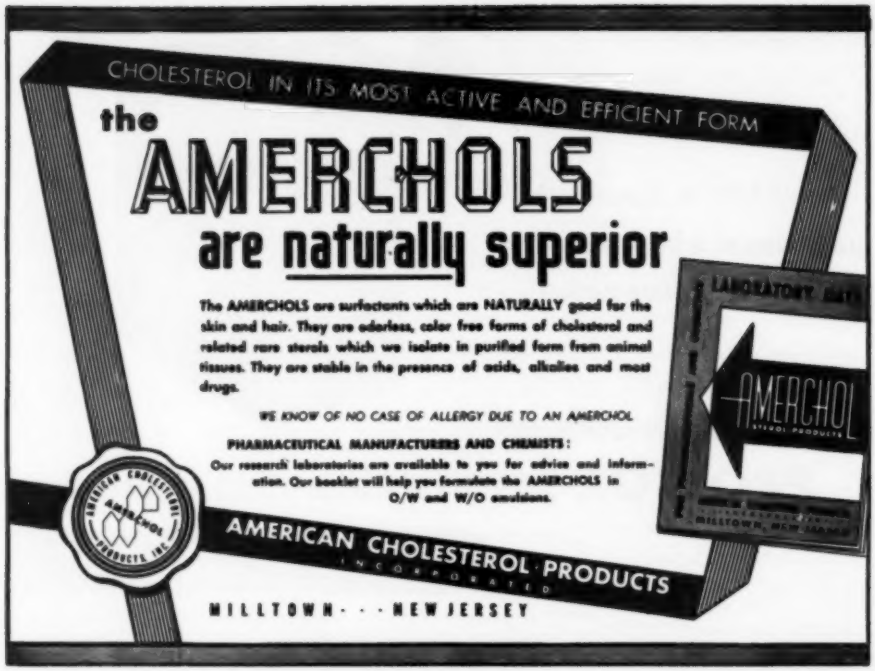
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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLI — *OCTOBER, 1952* — NUMBER 10 — CONSECUTIVE NO. 20

The Distribution of N^{15} in Rat Tissues Following the Intraperitoneal Administration of Nitrogen-Labeled Urethan*

By HAROLD S. BAILEY† and JOHN E. CHRISTIAN

A series of experiments designed to determine the distribution of N^{15} in the tissues and excretions of the rat following the intraperitoneal administration of nitrogen-labeled urethan is described and the results are reported. The concentration of N^{15} was generally higher in the bone marrow than in other tissues. The liver, gastrointestinal, spleen, and kidney proteins were found to contain greater concentrations than the brain, heart, lung, testes, skin, and muscle proteins. The amide nitrogen is excreted by way of the urine and feces. It is excreted in the urine partly as urea and partly as ammonium ion nitrogen.

URETHAN (ethyl carbamate) has long been known for its hypnotic properties. Recently, it was discovered that the administration of this drug to laboratory animals promoted the growth of pulmonary and hepatic tumors (1, 2). Subsequent experimentation showed that urethan possesses two other remarkable actions: the ability to retard the growth of specific tumors (3), and a destructive action on leucocytes (4).

Although still of doubtful clinical value, urethan has been used in the chemotherapy of various malignant conditions (5–8). The effects of its use in the chemotherapy of leukemia are qualitatively the same as those brought about by X-ray therapy in this disease. At the present time the administration of urethan is probably as good treatment as is available for multiple myeloma (8).

The many interesting pharmacological actions of urethan have served to initiate investigations into the mechanisms of action, detoxification, and metabolism of this important drug. There have been three studies reported concerning the distribution of the urethan molecule in the animal body. Bryan and his co-workers (9) studied the distribution of radioactivity in the tissues of mice following the injection of carbonyl-labeled urethan. The relative excretion of this portion of the molecule by the lungs and kidneys was also determined. Within twenty-four hours from the time of administration, 90 per cent of the radiocarbonyl carbon was accounted for in the respiratory carbon dioxide, and from 5 to 10 per cent of the total activity was found in the urine.

* Received April 4, 1952, from Purdue University, School of Pharmacy, Lafayette, Ind.
Presented before the Pharmacy Subsection, American Association for the Advancement of Science, Philadelphia meeting, December, 1951.
† Fellow of the American Foundation for Pharmaceutical Education, 1948–1950. Present address: Assistant Professor of Pharmacology, Division of Pharmacy, South Dakota State College, Brookings.

In 1949, Boyland and Rhoden (10) and, recently, Beickert (11) have investigated the distribution of the urethan molecule in the animal body. The results of these studies were similar in that the drug was found to be metabolized with only a small part of the administered dosage present in the tissues and excreted as urethan.

Due to the nearly complete catabolism of the drug, it was decided to make a study of the excretion and relative distribution of the amide nitrogen in the animal tissues. For this purpose, urethan labeled in the amide group with N^{15} was synthesized.

EXPERIMENTAL

The Synthesis of N^{15} Labeled Urethan.—The reaction between equimolar quantities of ethyl chloroformate, ammonia, and sodium hydroxide was found to be best suited for the synthesis of urethan labeled in the amide group with N^{15}.

$$CH_3CH_2{-}O{-}\overset{\overset{O}{\|}}{C}Cl + N^{15}H_3 + NaOH \rightarrow CH_3CH_2{-}O{-}\overset{\overset{O}{\|}}{C}{-}N^{15}H_2 + NaCl + H_2O$$

A solution of 4.49 Gm. of ammonium nitrate, labeled in the ammonium radical with 33.66 atom % N^{15} excess,[1] in 3 ml. of distilled, ammonia-free water was introduced all in one portion through a dropping funnel into a 50-ml. Claissen distilling flask which contained a boiling chip and 7.0 ml. of 50% NaOH solution. Two portions of 2 ml. each of ammonia-free distilled water were used to rinse the utensils and dropping funnel. The arm of the distilling flask was fitted with a condenser through which ice water was circulated. An adapter was adjusted to lead beneath the surface of 3 ml. of ammonia-free distilled water contained in a closed receiving tube, the outlet of which led to the surface of 5 ml. of 2 N H_2SO_4 contained in another 1 x 6-in. test tube. The receiving tube was immersed in ice water to cool the distillate further. The alkali-ammonia solution was heated to a bath temperature of 95° by means of an oil bath and the temperature was held between 95–100° for a period of fifteen minutes to allow the initial release of gas to proceed as slowly and smoothly as possible. The temperature of the oil bath was then gradually raised over a period of an hour to 140–150°. Eight milliliters of distillate was collected, after which heating was discontinued, the receiving vessel was detached from the apparatus, and the adapter was washed down with 2 ml. of distilled water. Preliminary runs yielded a solution of 0.955 Gm. of $N^{15}H_3$ by this method.

Ether (8 ml.) was added to the distillate contained in the 1 x 6-in. receiving tube. The tube was then fitted with a three-hole rubber stopper in which were inserted a glass stirring rod and two 5-ml. pipettes, calibrated after bending to contain 5.4 ml. (6.14 Gm. or 0.056 mole) of ethyl chloroformate in one pipette and 4.5 ml. of NaOH solution containing 0.5 Gm./ml. (2.25 Gm. or 0.056 mole) in the other pipette. The pipettes were fitted with rubber tubing and screw clamps, by means of which air could be admitted to the pipettes and, therefore, the rate of addition of the reactants regulated. The tube was then immersed in ice water and the reactants were added slowly, with constant stirring, to the solution containing 0.955 Gm. (0.056 mole) of $N^{15}H_4$. After all of the reactants were added, the ice bath was removed and stirring was continued for two hours, during which time the reaction mixture warmed up to room temperature.

The aqueous phase was then saturated with sodium chloride and the mixture was stirred for five minutes. The ethereal layer was drawn off by suction through a capillary tube into a 100-ml. round bottom flask. The process of extraction of the saturated salt solution was repeated four times with 10-ml. portions of ether, stirring for five minutes with each extraction. The ethereal extracts were combined in the 100-ml. round bottom flask and the ether was distilled off under reduced pressure through a series of three traps, consisting of 1 x 6-in. test tubes, two of which were empty while the middle trap contained 10 ml. of 2 N H_2SO_4. This apparatus was designed to catch any excess ammonia which might be present in the ethereal solution.

Ten milliliters of purified toluene and about 1 Gm. of anhydrous K_2CO_3 were added to the residue from the ethereal extract. After decantation, the dried toluene solution was then cooled in an ice bath, and the urethan crystallized. The product was recrystallized from toluene. A total of 4.23 Gm. of urethan labeled in the amide group with 33.66 atom % excess of N^{15} was obtained, representing 84.6% of the theoretical yield. The product melted at 47–48°, uncorrected (m. p. U. S. P. XIV, 48–50°).

The unreacted ammonia was recovered from a combination of the reaction residue, the residue from the ammonia distillation, and the acid traps used in the ammonia and ether distillation by alkalinizing the solution and distilling the ammonia into normal acid. Ammonia was also recovered by the Kjeldahl technique from the residue left after air evaporation of the toluene mother liquor. The total recovery of the ammonia was 92.75%.

Method of Analysis for Isotopic Nitrogen.—The method used for the conversion of organic nitrogen compounds to nitrogen gas was similar to that outlined by Rittenberg and his co-workers (12). The organic nitrogen compound was converted by a semimicro Kjeldahl procedure to an ammonium salt, following which the ammonium ion was oxidized to nitrogen gas by means of hypobromite. The resulting nitrogen gas was analyzed for isotopic content by means of a Consolidated-Nier Isotope Ratio Mass Spectrometer, Model 21-201.[2]

Studies on the Stability of the Carbon-Nitrogen Linkage in Urethan.—The validity of any conclusion drawn from investigations using the isotope tracer technique depends upon the stability of the molecule containing the labeled atom. If the carbon-nitrogen linkage in urethan is unstable, any conclusion as to the chemical removal of the amide group and its distribution in the animal body would not be valid.

[1] Atom % N^{15} excess = per cent of the total nitrogen atoms above the normal abundance (0.38%) which are N^{15} atoms.

[2] The authors gratefully acknowledge the assistance of Professor Egil Ramstad in the measurement of the isotope ratios.

The stability of this bond in the urethan molecule was determined by refluxing solutions of N^{15} labeled urethan (33.66 atom % N^{15} excess) and natural ammonium chloride and urea for eight hours. The isotopic concentration of the nitrogen from the separated, purified ammonium chloride and urea was then determined. It was concluded from the experimental results that negligible exchange exists between the nitrogen of urethan and ammonium ion or urea nitrogen at temperatures well above the physiological range.

The Distribution of N^{15} in Rat Tissues Following Intraperitoneal Administration of Nitrogen-Labeled Urethan.—This study was divided into the determination of the distribution of the amide nitrogen in rat tissues at three, twelve, and twenty-four hours from the time of injection. A study was also made of the distribution of the amide nitrogen in rat tissues twelve hours following the last of four successive injections of urethan at twelve-hour intervals.

Four groups of eight white, male (Purdue-Wistar strain) rats, whose average weight was approximately 250 Gm., were fasted for thirty-six hours. Water was permitted. An intraperitoneal injection of a single dose of N^{15}-labeled urethan (3.22 atom % N^{15} excess) equivalent to 1,000 mg./Kg. was given each animal in the first three groups. The eight animals in the fourth group were given intraperitoneal injections of the N^{15} labeled urethan (3.22 atom % excess) equivalent to 500 mg./Kg. every twelve hours for a total of four doses. The animals were placed in metabolism cages, and the urine and feces were collected. The urine of the eight animals in the fourth group was collected at twelve-hour intervals. At the end of three, twelve, and twenty-four hours, the animals in the first, second, and third groups, respectively, were killed by cardiac puncture, under ether anesthesia when necessary, and the blood was collected in oxalate. The animals in the fourth group were killed by cardiac puncture, under ether anesthesia, twelve hours following the last of the four successive injections given at twelve-hour intervals. From this point the experimental procedure was the same for all animal groups.

The contents of the gastrointestinal tracts were combined with the feces, and the various organs were isolated and worked up separately. Samples of the muscle, skin (razor shaved), and bone marrow were taken from each animal for investigation of their proteins.

The isotopic concentration of the nitrogen was determined in the following samples:

Excreta—Total urine, urine ammonia, urine urea, and feces.
Blood—Whole blood, whole plasma, plasma proteins, plasma NPN, corpuscles, and hemin.
Organs—Pooled organ NPN, the proteins of the brain, heart, lung, liver, gastrointestinal tract, spleen, kidney, and testes.
Carcass—Carcass proteins, carcass NPN, muscle proteins, skin proteins, and bone marrow.

Excreta.—The urine of all eight animals in a group was pooled and analyzed for isotopic concentration. Urine ammonia was isolated by absorption on acid-washed Permutite (13), and the isotopic concentration was determined. Urea was isolated as the dixanthydrol derivative (14), and a sample was used for isotopic analysis. The combined gastrointestinal tract contents and feces were digested and a sample was analyzed for N^{15} content.

Blood.—A sample of the pooled, oxalated blood from the eight animals in each group was analyzed for N^{15}. The remaining blood was centrifuged, the plasma was separated, and the cells were washed with isotonic saline and hemolyzed with water. Hemin was isolated from an acetic acid-sodium chloride solution (15). The plasma proteins were precipitated by the addition of five volumes of a 6% trichloroacetic acid solution. Samples of plasma, hemolyzed cells, hemin, plasma proteins, and plasma nonprotein nitrogen (NPN) were taken for analysis.

Organs.—The separately pooled brains, hearts, lungs, livers, washed gastrointestinal tracts, spleens, kidneys, and testes were minced and extracted with 5 times their weights of 6% trichloroacetic acid to separate the various organ proteins from the organ nonprotein nitrogen. The trichloroacetic acid extracts were combined and analyzed as organ NPN. Samples of the proteins of each of the pooled organs were analyzed for their isotopic nitrogen content.

Carcass.—The pooled, shaved skin samples were extracted with 5 times their weights of 6% trichloroacetic acid, and a portion of the insoluble proteins were analyzed for N^{15}. The muscle samples were treated in the same manner as the skin. The samples of the bone marrow taken from the rat femurs were pooled and digested directly for isotope determination.

After removal of the organs and samples of skin, muscle, and bone marrow, the remaining bodies of the eight animals were combined, cut up into pieces, and passed through a meat grinder twice. The mince was then extracted several times with 5 times its weight of 6% trichloroacetic acid. Samples of the proteins and the nonprotein nitrogen were taken for analysis.

Results.—The results of the analyses of samples obtained from the animal tissues after the injection of the labeled urethan are presented in Tables I and II. The isotopic concentration in excess of the natural abundance is expressed in two ways: first, the analytical value; and second, the value calculated on the basis of 100 atom-% excess N^{15} in the compound injected (atom % N^{15} calculated).

DISCUSSION

The results of this investigation substantiate the work of Bryan (9), Boyland and Rhoden (10), and Beickert (11) in that there is no indication of definite localization of the amide group and, therefore, of urethan in any one organ or tissue of the animal body. However, from this study we are unable to state whether there is a localization of a metabolite or metabolites of the drug in the tissues. Nevertheless, we can assume that there is negligible localization by the organs or tissues of any metabolite containing the amide portion of the molecule.

It will be noticed that there is a slight uptake of the amide nitrogen by all the body proteins analyzed. The organs directly concerned with metabolism and excretion were generally found to contain a greater quantity of the labeled nitrogen than the other organs.

TABLE I.—THE DISTRIBUTION OF N^{15} IN RAT TISSUES FOLLOWING INTRAPERITONEAL INJECTION OF LABELED URETHAN

	Single Injection, 1,000 Mg./Kg.						Multiple Injections[a]	
	Three Hours		Twelve Hours		Twenty-four Hours			
Source of N^{15}	Atom % N^{15}, Excess	Atom % N^{15}, Calcd.	Atom % N^{15}, Excess	Atom % N^{15}, Calcd.	Atom % N^{15}, Excess	Atom % N^{15}, Calcd.	Atom % N^{15}, Excess	Atom % N^{15}, Calcd.
$EtOCON^{15}H_2$	3.22	100.00	3.22	100.00	3.22	100.00	3.22	100.00
Blood, whole	0.010	0.31	0.035	1.07	0.048	1.49	0.024	0.75
Plasma, whole	0.057	1.77	0.072	2.24	0.037	1.15	0.097	3.01
Plasma, NPN[b]	0.468	14.53	0.250	7.76	0.209	6.49	0.452	14.20
Plasma proteins	0.006	0.19	0.008	0.23	0.023	0.71	0.056	1.74
Corpuscles	0.013	0.40	0.018	0.56	0.014	0.44	0.010	0.31
Hemin	0.005	0.15	0.007	0.22	0.008	0.25	0.024	0.75
Total urine	0.030	0.93	0.128	3.98	0.195	6.05	....	....
Urine ammonia	0.108	3.37	0.240	7.45	0.279	8.66	....	....
Urine urea	....	....	0.137	4.27	0.188	5.84	....	....
Feces	0.043	1.24	0.039	1.21	0.071	2.21	0.068	2.11
Carcass NPN	0.031	0.62	0.053	1.65	0.038	1.18	0.037	1.15
Carcass proteins	0.020	0.96	0.007	0.22	0.013	0.40	0.014	0.44
Muscle proteins	0.010	0.31	0.007	0.22	0.008	0.25	0.010	0.31
Skin proteins	0.006	0.19	0.007	0.22	0.010	0.31	0.010	0.31
Bone marrow	0.012	0.37	0.020	0.62	0.024	0.75	0.054	1.68
Organ NPN	0.043	1.34	0.057	1.77	0.068	2.11	0.114	3.48
Brain proteins	0.006	0.19	0.010	0.31	0.009	0.28	0.014	0.44
Heart proteins	0.012	0.37	0.009	0.28	0.008	0.25	0.015	0.47
Lung proteins	0.008	0.25	0.008	0.25	0.010	0.31	0.017	0.53
Liver proteins	0.005	0.16	0.015	0.47	0.022	0.68	0.037	1.15
Gastrointestinal protein	0.006	0.19	0.028	0.87	0.015	0.47	0.029	0.90
Spleen proteins	0.006	0.19	0.009	0.28	0.018	0.56	0.024	0.75
Kidney proteins	0.009	0.26	0.010	0.31	0.015	0.47	0.023	0.71
Testes proteins	0.005	0.15	0.015	0.47	0.011	0.34	0.015	0.47

[a] Distribution of N^{15} in the rat tissues twelve hours following four successive injections of labeled urethan (500 mg./Kg.) at twelve-hour intervals.
[b] NPN = Nonprotein nitrogen.

TABLE II.—DISTRIBUTION OF N^{15} IN URINE OF RATS GIVEN MULTIPLE INJECTIONS[a]

	Source of N					
	Total Urine		Urine Ammonia		Urine Urea	
Time, Hours[b]	Atom % N^{15}, Excess	Atom % N^{15}, Calcd.	Atom % N^{15}, Excess	Atom % N^{15}, Calcd.	Atom % N^{15}, Excess	Atom % N^{15}, Calcd.
Twelve	0.069	2.14	0.141	4.38	0.063	1.96
Twenty-four	0.184	5.72	0.234	7.27	0.165	5.13
Thirty-six	0.244	7.58	0.248	7.70	....	....
Forty-eight	0.258	8.02	0.262	8.14	....	....

[a] Distribution of N^{15} in the rat tissues twelve hours following four successive injections of labeled urethan (500 mg./Kg.) at twelve-hour intervals.
[b] Time intervals at which urine was collected from rats given four successive injections of 500 mg./Kg. labeled urethan at twelve-hour intervals.

When repeated doses are given over a period of time, the concentration of the amide nitrogen in the tissues is increased. Since, from previous work (9), it is known that the urethan molecule is readily broken down in the body, it may be assumed that the amide nitrogen from urethan present in the protein is either actually a part of the protein molecule or a urethan metabolite-protein combination. If the urethan molecule is acted upon by deamidases, then it is reasonable to assume that the amide nitrogen enters the metabolic pool and is utilized like the α-amino nitrogen of amino acids.

The plasma nonprotein nitrogen shows the greatest concentration of the labeled nitrogen due to the presence of metabolites such as urea and unmetabolized urethan. Of the other tissues, the bone marrow and plasma proteins contain the greatest concentration of N^{15}. This may be due to the high metabolic turnover of the tissues in the bone marrow and the greater concentration of N^{15} in the blood.

The excretion of the amide nitrogen is not entirely by way of the synthesis of urea, although it is evident that a large portion is excreted in this manner. The amide nitrogen is also excreted to a large extent in the urine in the form of ammonium ion nitrogen. Excretion also takes place through the intestine, as evidenced by the N^{15} content of the feces.

CONCLUSIONS

1. Urethan (ethyl carbamate) labeled in the amide group with N^{15} was synthesized.

2. In aqueous solution at 100°, the carbon-nitrogen linkage in urethan is stable. There is negligible exchange with ammonium or urea nitrogen under these conditions.

3. Studies of the distribution of the amide nitrogen from urethan in the rat following a single

intraperitoneal dose show that there is no localization of the amide nitrogen (and therefore urethan) in any particular organ or tissue of the body. There is a slightly greater concentration in the proteins of the liver, gastrointestinal tract, spleen, and kidney, than in the brain, heart, lung, testes, skin, and muscle proteins. The concentration of the amide nitrogen in the bone marrow was generally higher than that in the other tissues.

4. The distribution of the amide nitrogen from urethan twelve hours following a series of four successive intraperitoneal doses at twelve-hour intervals was similar to that found after a single dose; however, the concentrations found in the tissues were generally higher. The bone marrow and plasma proteins had the greatest concentrations of N^{15} other than that found in the plasma nonprotein nitrogen. The liver, gastrointestinal tract, spleen, and kidney had a much higher concentration of N^{15}, with the lung, testes, heart, brain, and skin having only a slight excess.

5. The amide nitrogen from urethan is excreted by way of the urine and feces. It is excreted in the urine partly as urea and partly in the form of ammonium ion nitrogen.

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The Action of Solubilizing Agents on the Solubility of Xanthine Derivatives in Water*,†

I. The Application of Partition Studies to Theophylline and Caffeine Combinations

By MARTIN BLAKE‡ and LOYD E. HARRIS§

The effect of solubilizing agents on the partition coefficient of caffeine and theophylline between chloroform and water was observed. Results indicate that caffeine and citric acid, caffeine and sodium acetate, caffeine and sodium benzoate, and theophylline and sodium acetate do not react while in solution. Caffeine and sodium salicylate, and theophylline and sodium salicylate, probably react by forming an associated product as a result of hydrogen bonding. Theophylline and ethylenediamine probably form a salt in solution, indicated by the large change in the coefficient of theophylline in the presence of ethylenediamine.

THE LOW SOLUBILITY of the xanthine derivatives in water necessitates the use of agents as solubilizers. Sodium acetate, sodium benzoate, and sodium salicylate are popularly used to solubilize caffeine. In addition to these salts, numerous amines are used to solubilize theophylline; the most popular is ethylenediamine. The fact that these combinations are inconsistently referred to as double salts, mixtures, or complexes indicates that their true nature is unknown. The diversity of opinion concerning the nature of these combinations is recognized in the United States Dispensatory (1).

Chambon, *et al.* (2), reported the formation of a complex between caffeine and sodium benzoate at room temperature. The complex consisted of 1 molecule of caffeine and 1 or 2 molecules

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of sodium benzoate. Above 37°, caffeine and sodium benzoate existed as a mixture of the components.

Nowatke (3) applied dialysis studies to combinations of caffeine with sodium benzoate, citric acid, and sodium salicylate; theobromine with sodium acetate, sodium benzoate, and sodium salicylate; and theophylline with sodium acetate. The dialysis constants of the components when in combination were found to agree with the constants of the individual components. The results indicated that the combinations studied were not complexes or compounds but were simple mixtures of the components.

Paul (4) and Paul and Ruhl (5) applied dialysis studies to similar combinations of xanthine derivatives and solubilizers. They confirmed the fact that no compound or complex is formed. A study of the absorption spectra also revealed that association had not taken place.

Kaufmann (6) studied the combination of calcium theobromine and calcium nitrite. Complex formation was shown to occur.

Neuberg (7) explained that salts in aqueous solutions exhibited what he termed a "hydrotropic phenomenon." These salts increased the solubility of numerous substances without necessarily reacting with them. An increase in solubility from 7 to 100 times the normal solubility could be realized.

Emery and Wright (8) studied the combination of caffeine with several solubilizing agents. Cryoscopic analysis and partition studies were employed. The presence of molecular compounds was reported when sodium salicylate and sodium benzoate were used to solubilize caffeine.

In the present study, the effect of various solubilizers was observed on the partition coefficient of theophylline and caffeine between chloroform and water. When a reaction occurred, there was a lowering of the coefficient in favor of the aqueous phase. Since the solubilizers used in this study were insoluble in chloroform, only the caffeine or theophylline which did not react with the solubilizer could distribute itself between the two immiscible phases. By varying the ratio of the solubilizer to xanthine derivative, the course of any reaction could be followed.

EXPERIMENTAL

Materials.—Caffeine and theophylline of U. S. P. quality were made anhydrous by drying in an oven at temperatures suggested in the U. S. P. The solvents used were redistilled chloroform saturated with triple distilled water and triple distilled water saturated with redistilled chloroform. Sodium salicylate, sodium acetate, and sodium benzoate (all C. P.) were used in this study, and all were dried to constant weight as suggested in the U. S. P. and N. F. The ethylenediamine was found to be 97.56% pure.

Procedure.—Approximately 200 mg. of caffeine or theophylline was accurately weighed and placed in a ground glass-stoppered bottle. A weighed aliquot of solubilizer was then added. For the caffeine combinations, 100-cc. bottles were used. To this was added 50 cc. of aqueous solvent, followed by 25 cc. of chloroform solvent. A total of 100 cc. of chloroform solvent and 50 cc. of aqueous solvent was used for the theophylline combinations. The bottles were stoppered and shaken vigorously for five minutes, and then were placed in a constant temperature water bath at 25° ± 0.3°, with intermittent agitation, for a period of two and one-half to three and one-half hours. The stopper was then removed from one of the bottles, and while the bottle was still in the water bath, a suitable aliquot was removed from the chloroform layer. Twenty cubic centimeters was removed from each of the caffeine solutions and 25 cc. from each of the theophylline solutions. The aliquots were placed in tared beakers, evaporated to dryness on a steam bath, and dried to constant weight in an oven. From the original weight of xanthine derivative and from the weight in the chloroform aliquot, it was possible to calculate the partition coefficient. The residues from the chloroform aliquots were analyzed by taking a melting point and a mixed melting point with the respective xanthine derivative. The residues were further tested for the presence of any solubilizer which may have combined with xanthine derivative and thereby be distributed in the chloroform layer. U. S. P. official tests were used for the identification of the ions.

RESULTS AND DISCUSSION

An examination of the data shows no reaction in the cases of caffeine and citric acid, caffeine and sodium benzoate, theophylline and sodium acetate, and caffeine and sodium acetate. The partition coefficient remained constant within experimental error. However, in the cases of caffeine and sodium salicylate, theophylline and sodium salicylate, and theophylline ethylenediamine, there was evidence of a reaction taking place, indicated by a change in coefficient with increase in concentration of solubilizer.

Figure 1 shows a plot of the mole ratio of sodium salicylate to caffeine against the observed partition coefficient. The coefficient decreased from a maximum value of 21.57 to a minimum value of about 8.

Figure 2 shows a plot of the mole ratio of sodium salicylate to theophylline versus the observed partition coefficient. There was a decrease in the coefficient from a value of 0.350 to a low of 0.205.

The mole ratio of ethylenediamine to theophylline was plotted against the observed partition coefficient in Fig. 3. A characteristic decrease in coefficient from 0.350 to 0.00745 was produced.

The difference in the results for the sodium salicylate combinations as compared with the sodium benzoate combinations can be explained by

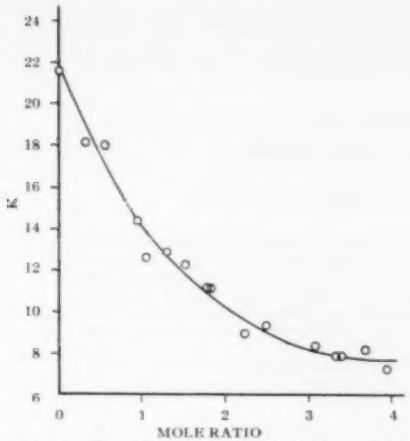

Fig. 1.—The effect of sodium salicylate on the distribution coefficient of caffeine between chloroform and water.

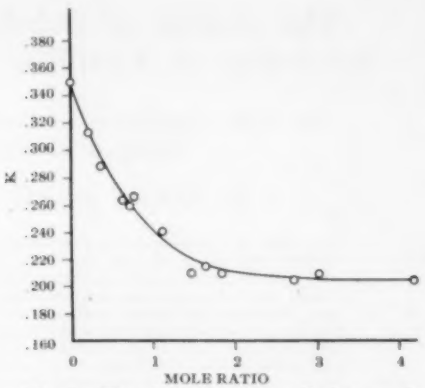

Fig. 2.—The effect of sodium salicylate on the distribution coefficient of theophylline between chloroform and water.

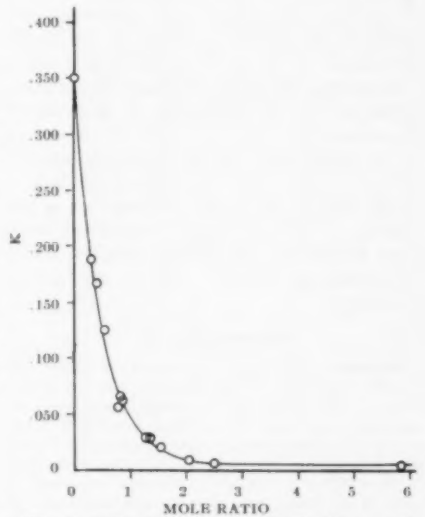

Fig. 3.—The effect of ethylenediamine on the distribution coefficient of theophylline between chloroform and water.

hydrogen bonding which results in an association between the hydroxyl of the salicylate and the carbonyl of the caffeine or theophylline molecule. Since the benzoate ion contains no phenolic hydroxyl group, this precludes the formation of an associated product.

The results obtained in this study present evidence for the formation of a complex between caffeine and sodium salicylate. Howeves, it appears that caffeine and sodium benzoate do not react in the manner reported by Emery and Wright (8).

The nature of theophylline ethylenediamine is particularly confusing in the literature. The large decrease in the partition coefficient of theophylline observed in this study is probably the result of salt formation. It is apparent that the combination is not merely a mixture as so often referred to in the literature.

SUMMARY

1. A method for the investigation of caffeine and theophylline combinations is presented. The method is based on partition studies and permits the detection of reaction between the solubilizer and the xanthine derivative.

2. Caffeine and sodium acetate, caffeine and citric acid, caffeine and sodium benzoate, and theophylline and sodium acetate do not react while in solution. These combinations are only mixtures.

3. Caffeine and sodium salicylate as well as theophylline and sodium salicylate react probably by forming an associated product as a result of hydrogen bonding. This may not necessarily be responsible for the solubilizing action of sodium salicylate. These combinations appear to be complexes.

4. The effect of ethylenediamine on the partition coefficient of theophylline is probably the result of the formation of a salt.

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The Action of Solubilizing Agents on the Solubility of Xanthine Derivatives in Water*,†

II. The Application of Conductimetric Titrations to the Study of Xanthine Combinations

By MARTIN BLAKE‡ and LOYD E. HARRIS§

The effect of xanthine derivatives on the conductance of the solubilizing agent in solution was observed. No reaction could be noted when citric acid was titrated with caffeine, sodium acetate with caffeine, sodium benzoate with caffeine, sodium acetate with theobromine, sodium benzoate with theobromine, sodium salicylate with theobromine, sodium acetate with theophylline, sodium benzoate with theophylline, or sodium salicylate with theophylline. In the case of caffeine and sodium salicylate there was a progressive decrease in conductivity. The curves obtained with theophylline ethylenediamine as well as theobromine ethylenediamine represent typical acid-base reactions.

THE NATURE of the combinations in which caffeine, theophylline, and theobromine are combined with solubilizing agents is not clearly understood, as indicated in Part I of this series. In the present study, the influence of the xanthine derivatives on the conductance of solutions of the solubilizing agents is observed. A change in the conductance of an electrolyte solution when a nonelectrolyte is added results from a change both in the number of ions and in the mobility of the ions present. This is indicative of an interaction. If reaction does not occur, the conductance of the solution will remain unchanged.

EXPERIMENTAL

Apparatus.—The apparatus used in this study for the conductance measurements was a modified form of the Leeds and Northrup Company conductance apparatus for general laboratory work and is fully described in their catalog (1). Additional capacitors were placed in the circuit to make a closer balance, and an oscillograph was used in place of the telephone as a null-point indicator. A narrow trace appeared on the screen of the oscillograph when balance was achieved. This represented a weak current flowing across the bridge. The conductivity cell was a typical cell used for titrations.

Materials.—Caffeine and theophylline, U. S. P., both anhydrous; theobromine, N. F., anhydrous; sodium salicylate, sodium acetate, sodium benzoate, and citric acid, all C. P. and dried to constant weight, were used in these experiments. Ethylenediamine, 97.56% pure, was also used.

Procedure.—A solution of known concentration of the solubilizing agent was diluted to 100 cc. and placed in the conductivity cell. The electrolyte was used as the solution in the cell because it was easier to detect compound or complex formation when a nonelectrolyte was added to a solution of a strong electrolyte. Any deviation from a straight line was indicative of reaction.

The sensitivity of the pattern on the oscillograph screen was kept low by turning down the volume of the amplifier. The bridge was brought to a state of balance by adjustment of the resistance box in such a way as to diminish the height of the pattern on the oscillograph. The volume control on the amplifier was then turned up and the bridge was balanced again more closely. When the sensitivity of the volume control was at its maximum volume, finer adjustments were made by increasing the frequency on the oscillator. This procedure was followed after each addition of titrant. The titrant, added in the form of the anhydrous powder, was weighed on an analytical balance and transferred to the cell. This procedure eliminated the dilution effect of the titrant and was found suitable for the low solubility of the xanthine derivatives. If, after forty-five minutes, solubility was not effected, the titration was consummated. Conductivity values were plotted against mg. of titrant added. These results are shown in Figs. 1–5.

RESULTS AND DISCUSSION

No change in conductivity was observed when citric acid was titrated with caffeine, sodium acetate with caffeine, sodium benzoate with caffeine, sodium acetate with theobromine, sodium benzoate with theobromine, sodium salicylate with theobromine, sodium acetate with theophylline, sodium benzoate with theophylline, or sodium salicylate with theophylline. On the basis of the curves obtained (Figs. 1 and 2), it can be said that the increased solubility of the xanthine derivatives produced by the solubilizer in water is not due to complex or compound formation.

In the case of caffeine and sodium salicylate, Fig. 3, there was a progressive decrease in the conductivity. This may have been produced by the

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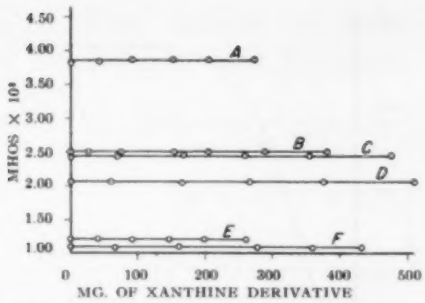

Fig. 1.—The effect of xanthine derivatives on the conductance of organic salts.

A. The effect of theobromine on 129.4 mg. of sodium acetate.
B. The effect of theobromine on 127.0 mg. of sodium salicylate.
C. The effect of caffeine on 120.7 mg. of sodium benzoate.
D. The effect of theophylline on 116.7 mg. of sodium salicylate.
E. The effect of theobromine on 76.5 mg. of sodium benzoate.
F. The effect of theophylline on 62.3 mg. of sodium benzoate.

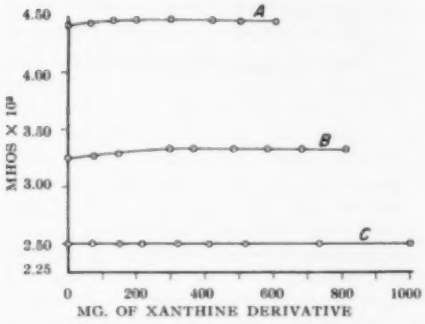

Fig. 2.—The effect of xanthine derivatives on the conductance of organic salts.

A. The effect of theophylline on 122.3 mg. of sodium acetate.
B. The effect of caffeine on 186.6 mg. of sodium acetate.
C. The effect of caffeine on 153.4 mg. of citric acid.

formation of new ions having a mobility slower than those originally in solution. Since no change was observed in the case of the titration of sodium benzoate with caffeine, it is possible that the phenolic hydroxyl group of the salicylate ion is capable of forming hydrogen bonds with the carbonyl oxygen of the caffeine molecule. This produces an associated product and a consequent decrease in conductivity.

Partition studies (2) showed that an interaction occurred between theophylline and sodium salicylate, but to a lesser extent than between caffeine

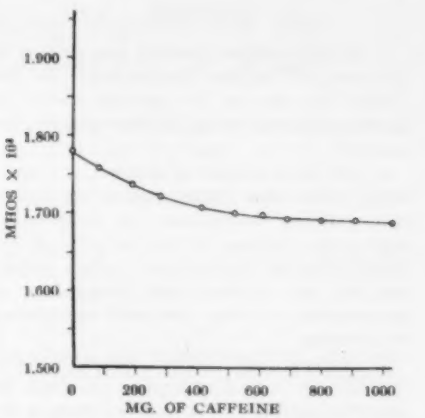

Fig. 3.—The effect of caffeine on the conductance of 112.8 mg. of sodium salicylate.

and sodium salicylate. It appears that conductimetric titrations were not as sensitive as partition studies in detecting this interaction.

The titration curves of theophylline and ethylenediamine, Fig. 4, and theobromine and ethylene-

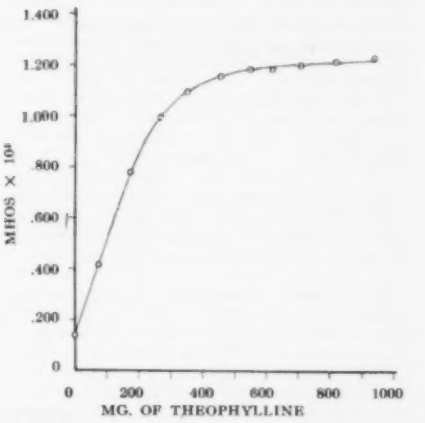

Fig. 4.—The effect of theophylline on the conductance of 98.7 mg. of ethylenediamine.

diamine, Fig. 5, represent typical acid-base reactions. The salt formed in either case is a salt of a very weak acid and a weak base. The curves are analogous to that shown by Britton (3) for the titration of boric acid with ammonium hydroxide. The pK values correspond to those for the acid and base used in this study. In any case, a rounded curve is obtained with no observable end point. Since an excess of base is required to completely neutralize the very weak acid, theophylline, an excess of ethylenediamine serves to stabilize the salt, theophylline ethylenediamine.

SUMMARY

1. Conductimetric titrations were applied to the study of xanthine combinations. By this method the effect of the xanthine derivatives on the conductance of the solubilizing agents was analyzed.

2. No reaction could be detected by conductivity studies when sodium acetate was titrated with caffeine, theophylline, or theobromine; when sodium benzoate was titrated with caffeine, theophylline, or theobromine; when sodium salicylate was titrated with theophylline or theobromine; or when citric acid was titrated with caffeine.

3. Caffeine and sodium salicylate react, probably by hydrogen bonding, to form an associated product. This is indicated by a decrease in conductivity when sodium salicylate is titrated with caffeine. This combination is best referred to as a complex. The method used was probably not sensitive enough to detect hydrogen bonding in the cases of theophylline and theobromine with sodium salicylate.

4. The titration curves for theophylline and ethylenediamine, and theobromine and ethylenediamine represent typical curves for the titration of a weak base with a very weak acid. These combinations are probably salts.

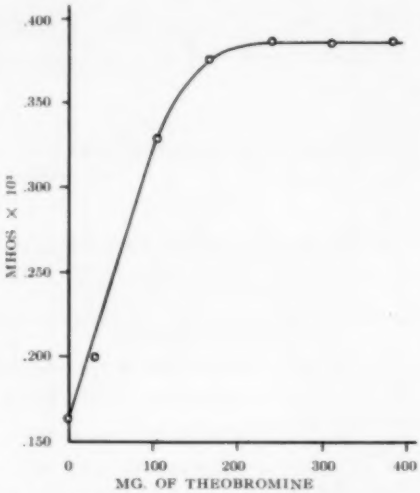

Fig. 5.—The effect of theobromine on the conductance of 65.5 mg. of ethylenediamine.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Xanthotoxin
D-Tagatose
Steapsin
Pyrithiamine
Sodium choleate
Oxythiamine
Methoxy-DL-tyrosine
Enterokinase
Anserine
p-Aminogentisic acid
Atropic acid
Brassylic acid
Cerotic acid
Desoxyphylloerythrin
2,9-Dimethyl-1,10-phenanthroline
Dithioglycol
Ethyldimethylcetylammonium chloride
2-Imidazolidone
Monocetyl phosphate
1-Phenyl-2,5-dimethyl-1,2,4-triazole

The Action of Solubilizing Agents on the Solubility of Xanthine Derivatives in Water*,†

III. The Application of Cryoscopy to the Study of Caffeine, Theophylline, and Theobromine with Solubilizing Agents

By MARTIN BLAKE‡ and LOYD E. HARRIS§

Cryoscopy was applied to the study of xanthine combinations for the purpose of detecting interaction between the xanthine derivatives and the solubilizing agent. Caffeine, theophylline, and theobromine react with sodium salicylate probably by means of hydrogen bonding to form an associated product. The combination of theophylline, theobromine, or caffeine with sodium acetate shows no indication of reaction. The same is true of combinations with sodium benzoate.

SINCE the depression of the freezing point produced by solutes in water depends on the number of particles in solution, cryoscopic measurements can be used to detect reaction between solutes. If reaction does not take place, the lowering of the freezing point will be the additive value of the individual solutes. If reaction does take place, the lowering of the freezing point will depend on the number of particles present as the result of the reaction. This study was undertaken to elucidate further the interaction between xanthine derivatives and solubilizing agents. The combinations of caffeine, theophylline, and theobromine with sodium benzoate, sodium acetate, and sodium salicylate were considered. The method described by Daniels, Mathews, and Williams (1) was employed for this study. The precautions discussed by Findlay (2) were carefully considered.

EXPERIMENTAL

Apparatus.—A test tube of 50-cc. capacity which served as the freezing-point tube was passed through a cork stopper into a larger test tube. The space between the two test tubes served as an air mantle and permitted a slow change in temperature. This system was placed in a 2,000-cc. beaker which served as a cooling bath. Crushed ice and commercial sodium chloride composed the freezing mixture, the temperature of which was maintained from 3–5° below the freezing point of the solution. A Beckmann thermometer and a glass stirrer were placed in the freezing-point tube, and a thermometer graduated from 50° above zero to 50° below zero was placed in the cooling bath.

Materials.—Anhydrous caffeine and theophylline of U. S. P. quality, theobromine of N. F. quality, and sodium salicylate, sodium acetate, and sodium benzoate, all of C. P. quality, dried as suggested in the U. S. P. and N. F., were used.

Procedure.—The ice bath was prepared with sufficient commercial sodium chloride to produce a temperature of −4°. Twenty cubic centimeters of water was added to the freezing-point tube which was then placed directly in the ice bath with the stirrer and Beckmann thermometer affixed. When the temperature had fallen to 0.5°, the freezing-point tube was then placed in the air mantle. The solution was stirred slowly and the temperature was observed until a constant reading was obtained. This temperature was the observed freezing point of water.

In a similar manner, 20 cc. of a solution of known concentration of solubilizing salt was added to the cleaned and dried freezing-point tube, and its freezing point was determined. The tube was removed from the mantle and warmed until all the ice had melted. Ten cubic centimeters of the solution was then removed by pipette and placed in a tared beaker. The beaker and its contents were immediately weighed. The solution was then evaporated to dryness in an oven and dried to constant weight, cooled, and reweighed. From these data the theoretical depression of the freezing point was calculated and compared with the observed value.

An amount of the xanthine derivative in excess of the normal solubility of the compound in water was mixed with a solution of salt of the same concentration as previously studied. The mixture, in a suitable container, was placed in the cooling bath; when several degrees above the freezing point, it was removed from the cooling bath and filtered. A portion of 20 cc. of the filtrate was removed by pipette and placed in the cleaned and dried freezing-point tube. The freezing point of the solution was determined as described previously. The freezing-point tube was removed from the air mantle and warmed until all the ice melted. An aliquot of 10 cc. of the solution was removed by pipette and placed in a tared beaker. The beaker was immediately weighed, and the solution was evaporated to dryness and dried to constant weight in an oven. Since the amount of salt in the residue was known, the weight of the xanthine derivative was calculated by difference. From the data, the theoretical increase in the freezing-point depression produced by the xanthine derivative was calculated. This was compared with the observed depression.

* Received November 30, 1951, from the College of Pharmacy, Ohio State University, Columbus.

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TABLE I

Salt Soln. Concn.	Xanthine Derivative Concn.	F. P. Depression by Salt	F. P. Depression by Xanthine Derivative	Theoretical Depression by Xanthine Derivative
		Caffeine and Sodium Salicylate		
0.100	0.113	0.330	−0.004	0.210
0.100	0.170	0.375	−0.002	0.316
0.100	0.0551	0.378	−0.004	0.102
		Theophylline and Sodium Salicylate		
0.100	0.0833	0.383	−0.007	0.155
0.100	0.0486	0.379	−0.004	0.090
0.100	0.0104	0.374	−0.008	0.019
		Theobromine and Sodium Salicylate		
0.500	0.0948	1.804	−0.003	0.176
0.500	0.0441	1.794	−0.009	0.082
0.500	0.0761	1.832	−0.004	0.142
		Caffeine and Sodium Acetate		
0.500	0.0973	1.859	0.172	0.180
0.500	0.176	1.721	0.295	0.327
0.500	0.243	1.740	0.435	0.452
		Theophylline and Sodium Acetate		
0.500	0.0555	1.682	0.098	0.103
0.500	0.0324	1.753	0.058	0.060
0.500	0.0166	1.818	0.023	0.030
		Theobromine and Sodium Acetate		
0.500	0.0843	1.763	0.146	0.157
0.500	0.0532	1.812	0.092	0.099
0.500	0.0247	1.799	0.044	0.046
		Caffeine and Sodium Benzoate		
0.100	0.142	0.368	0.264	0.264
0.100	0.104	0.375	0.187	0.193
0.100	0.217	0.371	0.397	0.404
		Theophylline and Sodium Benzoate		
0.500	0.0771	1.884	0.145	0.143
0.500	0.0411	1.832	0.073	0.076
0.500	0.0121	1.791	0.022	0.023
		Theobromine and Sodium Benzoate		
0.500	0.0241	1.923	0.047	0.045
0.500	0.0116	1.786	0.018	0.022
0.500	0.0097	1.794	0.018	0.018

RESULTS AND DISCUSSION

In Table I, the molal concentration of the solubilizing salt is given in column 1, the concentration of xanthine derivative in column 2, the freezing point depression produced by the salt in column 3, the effect of xanthine derivative on the freezing-point depression of the salt in column 4, and the theoretical molal freezing-point depression of the xanthine derivative, if it had been alone in solution, in column 5. All freezing-point depressions reported in Table I represent the average of three determinations, each of which varied from the mean value by no more than 0.003°.

From Table I, it is seen that there is no further increase in the freezing-point depression when caffeine is added to the sodium salicylate solutions and thus no further increase in the number of particles present in solution. The same situation is found to exist when theophylline or theobromine is added to sodium salicylate solutions, indicating the formation of an addition product, probably by means of hydrogen bonding between the phenolic hydroxyl of the salicylate and the carbonyl oxygen of the xanthine derivative. As a result, the number of particles in solution is the same as that for the sodium salicylate alone.

In the cases of combinations of sodium acetate with caffeine, theophylline, or theobromine, as well as the combinations of sodium benzoate with caffeine, theophylline, or theobromine, the calculated depression of the freezing point as well as the theoretical depression of the freezing point agree within experimental error. This indicates that, when the xanthine derivative is added to the aqueous solution of the salt, there is an increase in the freezing-point depression proportional to the molal concentration of the xanthine derivative. This is expected if reaction does not occur.

The results of cryoscopic studies further substantiate the formation of a complex between caffeine and sodium salicylate as observed by Emery and Wright (3). However, contrary to their findings, there does not appear to be any interaction between caffeine and sodium benzoate.

SUMMARY AND CONCLUSIONS

1. Cryoscopy was applied for the purpose of detecting any reaction between xanthine derivatives and solubilizing agents. The freezing point of a solution of known concentration of solubilizer was noted, and the effect of the xanthine derivative in depressing the freezing point of the solution of the solubilizer was then observed.

2. No further depression in the freezing point was observed when caffeine, theophylline, or theobromine was added to a solution of sodium

salicylate. This indicates the formation of an associated product, probably as a result of hydrogen bonding.

3. In combinations of sodium acetate with caffeine, theophylline, or theobromine, the calculated and theoretical depression of the freezing point agreed within experimental error. The same was true with combinations of sodium benzoate with caffeine, theophylline, or theobromine.

4. There was an increase in the freezing point depression which was proportional to the molal concentration of the xanthine derivative. This would be expected if reaction did not occur.

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(2) Findlay, A., "Practical Physical Chemistry," Longmans, Green and Co., London, 1941, p. 127.
(3) Emery, W. O., and Wright, C. D., *J. Am. Chem. Soc.*, **43**, 2323(1921).

The Composition of Silk Oak Gum*

By ERNEST ANDERSON† and LELAND HARRIS‡

Silk oak gum is a mixture of polyuronides which differ slightly in their composition and solubility in water. The polyuronides consist of the calcium and magnesium salts of D-glucuronic acid to which are attached D-galactose and L-arabinose. The purest samples of the crude gum analyzed contained approximately 2.66 units of anhydro D-galactose and 2.23 units of anhydro L-arabinose for each unit of D-glucuronic acid. A mild acid hydrolysis liberates all of the L-arabinose but leaves most of the D-galactose attached to the D-glucuronic acid. More vigorous acid hydrolysis liberates all but one D-galactose unit and leaves an aldobionic acid consisting of D-glucuronic acid combined with one unit of D-galactose.

THE TREE *Grevillea robusta* (A. Cunn) is a native of the coastal regions of Queensland and New South Wales, Australia, where it is called silky oak (1) or lacewood. It belongs to the family *Proteaceae* and is a valuable commercial wood. It has been introduced as an ornamental tree into the southern United States from Florida to California, where it is known as silk oak.[1]

EXPERIMENTAL

Source.—The gum used in this investigation was collected from trees growing in Phoenix, Arizona.[2] Most of it was from trees diseased or injured in some way. Healthy trees did not contain much gum, which supports the suggestion that plant gums are usually pathological products of plant metabolism. However, silk oak is said to be very susceptible to gummosis (2).

Purification.—Samples of the gum varied in color from dark brown to light reddish brown. The gum was purified by heating it with 20 times its weight of water in a steam bath. A milky, colloidal solution resulted which, while hot, was pressed through cloth. The cold solution was added to approximately 8 times its volume of ethanol. A small amount of sodium chloride was added to flocculate the solution. After the precipitate had settled, the solvent was decanted and the precipitate was rubbed with ethanol. A light-colored, granular solid resulted, which was a mixture of polyuronides that differ in their solubility in water.

Fractionation.—When some of the purified gum was heated with 60 times its weight of boiling water and the hot solution was filtered through cloth and filter paper, approximately 20% remained undissolved. When this insoluble portion (Fraction B) was rubbed with ethanol, it formed a granular solid.

The aqueous filtrate from Fraction B was treated for a short time with a small amount of liquid bromine, and then 50 times its volume of ethanol was added. This precipitated Fraction A_1 which amounted to approximately 70% of the gum used.

When more ethanol was added to the filtrate from Fraction A_1, a second precipitate, Fraction A_2, slowly formed, amounting to approximately 10% of the gum used; $[\alpha]_D^{25} = -8.1$.

Preparation of the Free Gum Acid.—A colloidal solution of the crude gum in hot water was pressed through cloth. The cold solution was made 1.5% acid with hydrochloric acid, and was immediately diluted with 8 volumes of cold ethanol to precipitate the gum acid. After washing with ethanol and drying, the yield was 80% of the gum used.

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[1] Eloise Gerry of the Forest Products Laboratory, Madison, Wisc., supplied some information relative to the silk oak tree.
[2] Our attention was first called to this gum in 1941 by S. B. Detwiler of the U. S. Department of Agriculture.

TABLE I.—ANALYSIS OF UNPURIFIED AND PURIFIED SILK OAK GUM AND GUM ACID[a]

	Crude Gum	Fraction A_1	Fraction A_2	Fraction B	Gum Acid
Moisture, %	11.87	5.27	5.29	5.37	6.68
Ash, %	3.03	1.13	0.00	2.03	0.22
Carbon dioxide, %	4.79	4.42	4.18	4.58	3.74
Uronic acid, %	21.11	19.49	18.43	20.19	16.49
L-Araban, %	31.96	28.48	30.03	25.08	31.66
D-Galactan, %	47.03	52.13	51.64	54.83	51.94
Equivalent weight from uronic acid	919.00	995.00	1053.00	961.00	1176.00
Araban units per uronic acid	2.23	2.15	2.39	1.83	2.82
Galactan units per uronic acid	2.66	3.20	3.35	3.25	3.77

[a] The results are corrected for moisture and ash. The pentosan was calculated as araban; the per cent galactan was calculated as the difference between 100 and the sum of the percentages of uronic acid and araban.

Solubility.—When powdered samples (0.1 Gm.) of crude gum, the gum acid, and Fractions A_1 and B were each shaken with 100 ml. of water at 25°, they slowly swelled somewhat but did not dissolve. On the other hand, Fraction A_2 dissolved under these conditions.

Analysis of the Gum.—The presence of a uronic acid in the gum was established by the naphthoresorcinol test (3, 4) and by the evolution of carbon dioxide when the gum was heated with a 12% solution of hydrochloric acid (5). Tests for methoxyl groups by the method of Denigès (6) and von Fellenberg (7) were negative.

The results of the analyses of the crude gum, the gum acid, and Fractions A_1, A_2, and B are given in Table I. The methods used in the analyses are standard and are fully described in the literature (3, 8, 9). The results are corrected for moisture and ash. Since it is shown below that L-arabinose and D-galactose are the only sugars present, the percentages of pentosan and hexosan are reported in Table I as araban and galactan. The percentages of D-galactan may be somewhat high since they were calculated by subtracting the sum of the percentages of the uronic acid and L-araban from 100.

The analyses show that in the crude gum there are 2.23 araban groups and 2.66 galactan groups to each uronic acid group. After fractionation of the gum, the relative number of galactan groups to each uronic acid group increases, while the number of araban groups decreases. This is probably due to slight hydrolysis of arabinose during the process of fractionation.

Hydrolysis of the Gum.—A number of samples of the gum were hydrolyzed under varying conditions. Weighed samples were mixed with a known volume of water and sulfuric acid and the mixture was heated for a known length of time and at a known temperature. The solution was then neutralized by addition of barium carbonate, decolorized with activated carbon, and filtered at the pump; the filtrate was then concentrated *in vacuo* to a thin syrup. The barium salts were precipitated by addition of the syrup to ethanol, and were further purified by redissolving them in water and adding the solution to ethanol. The alcohol solutions of the sugars, free of barium salts, were concentrated *in vacuo* to syrups. In this way the salts were separated from the sugars.

Identification of L-Arabinose and D-Galactose.—Pentosan determinations and tests for pentoses and methyl pentoses showed the presence of large amounts of a pentose (3, 8, 9) and the absence of methyl pentoses. It is known (10) that during long heating of a polyuronide in acid solution much of the uronic acid is destroyed and the sugars combined with it are liberated. Hence, the sugar solutions obtained from the various acid hydrolyses of this gum would contain some of all the sugars present in it.

The sugar syrups, obtained from the various hydrolyses of the gum, as previously described, were used in identifying the sugars present. In all cases when these sugar syrups were dissolved in glacial acetic acid and allowed to stand, crystalline sugars were obtained whose specific rotations varied from $[\alpha]_D^{25} = +81°$ to $+96°$. These crystals were found to be mixtures of D-galactose, $[\alpha]_D^{25} = 80.5°$, and L-arabinose, $[\alpha]_D^{25} = +104.5°$. The D-galactose was identified by oxidation with a 20% solution of nitric acid and by the isolation of insoluble mucic acid melting at 214°. The L-arabinose was identified by conversion to the diphenyl hydrazone melting at 196° and also to the benzyl phenyl hydrazone melting at 174°. The paper chromatographic method of Rao and Beri (11) also showed the presence of these two sugars and no others.

The syrups remaining after removal of the crystalline D-galactose and L-arabinose gave the same positive tests for these two sugars. Fermentation tests with ordinary baker's yeast were negative, showing the absence of D-glucose, D-fructose, and D-mannose. Bertrand's test (3) for D-xylose was also negative. The only sugars present are D-galactose and L-arabinose.

Identification of D-Glucuronic Acid.—The presence of D-glucuronic acid was established by oxidizing (with 20% nitric acid) an aldobionic acid prepared from the gum, which consisted of the uronic acid combined with D-galactose. After removal of the insoluble mucic acid formed from the D-galactose, saccharic acid was isolated from the oxidation mixture as potassium acid saccharate. An analysis of this showed $[\alpha]_D^{25} = +4.00$; the silver salt showed 49.00% silver, which could only be formed if D-glucuronic acid were present, since the absence of D-glucose had already been established.

A spectroscopic analysis of the ash showed the presence of large amounts of calcium and magnesium and no more than traces of other metals. The gum consists of the calcium and magnesium salts of D-glucuronic acid which is combined with D-galactose and L-arabinose.

Analysis of the Barium Salts.—The results of the analysis of the various barium salts obtained by hydrolysis of the gum as previously outlined are

TABLE II.—ANALYSIS OF BARIUM SALTS FROM HYDROLYSIS OF SILK OAK GUM[a]

	Salts from Gum Heated for 2.5 Hr. at 80°; Barium Salt 1	Salts from Gum Heated for 3.5 Hr. at 80°; Barium Salt 2	Salts from Gum Heated for 13 Hr. at 98°; Barium Salt 3	Salts from Gum Heated for 15 Hr. at 98°; Barium Salt 4	Salts from Gum Heated for 18 Hr. at 98°; Barium Salt 5	Salts from Barium Salts 3 and 5 Heated for 6 Hr. at 98°; Barium Salt 6
Barium, %	7.29	9.79	13.18	18.12	15.55	15.40
Carbon dioxide, %	7.78	7.89	8.84	9.59	10.68	10.01
Uronic acid, %	34.13	34.61	38.78	42.06	46.85	43.80
Equivalent weight	566.00	558.00	498.00	459.00	412.00	440.00
Anhydro hexose units per uronic acid	1.88	1.83	1.46	1.22	0.93	1.11
Specific rotation, $[\alpha]_D$	+57.8°	+50.3°	−12.5°	...	−19.4°	−19.2°

[a] Barium salts 1, 3, 4, and 5 were from gums hydrolyzed in a 4% solution of sulfuric acid. Barium salts 2 and 6 were from gums hydrolyzed in 3.5% and 2% solutions of sulfuric acid, respectively. No pentosans or anhydro pentose units were present.

given in Table II. In all the analyses of the barium salts, only small amounts of furfural were obtained and these corresponded to the furfural that would be given by the uronic acid present. All the salts were free of any pentose. In every case all of the L-arabinose originally present in the gum had been liberated during hydrolysis. The ease with which this sugar was liberated suggests that it was present in the gum in the furanose form.

The results given in columns 1 and 2 of Table II show that when the gum is heated to 80° for two and one-half hours in a 4% solution of sulfuric acid, all of the L-arabinose groups and some of the D-galactose groups are removed. Slightly less than two galactose groups remain for each uronic acid group. The results listed in columns 3 and 4 show that when the hydrolysis is carried out at a higher temperature and for a longer period in 4% sulfuric acid, fewer galactose groups remain attached to the uronic acid. The results given in columns 5 and 6 show how difficult it is to liberate the last galactose unit from the uronic acid. This difficulty in liberating the last sugar molecule that is attached to a uronic acid has previously been reported (9).

The changes in the specific rotation of the salts during progress of the hydrolysis are particularly significant. They change from strongly dextro when hydrolyzed for a short time to strongly levo when hydrolyzed more drastically. The great difference in the rotation of the salts shown in columns 1 and 2 as compared with those listed in columns 5 and 6 suggests that a profound change occurred in the structure of the salts during the prolonged heating at a higher temperature, indicating that the structure of this gum resembles that of some other plant gums in that it is composed of branching chains of polyuronides combined together. During early stages of hydrolysis, the chief change is the liberation of sugar groups which are attached to the outer fringe of the molecule, leaving largely unchanged, however, the nucleus of the molecule which is composed of combined polyaldobionic acids. Under the longer and more drastic conditions of continued hydrolysis, this nucleus is gradually split apart into simple aldobionic acids. The salts shown in columns 1 and 2 are probably those of complex polyaldobionic acids, while those in columns 5 and 6 are of simple aldobionic acids containing one uronic acid group combined with one D-galactose group.

SUMMARY

The silk oak tree, *Grevillea robusta*, is an Australian tree that has been introduced into the southern United States. The gum used in this investigation was collected from trees growing in Phoenix, Arizona. This gum, which is only partially soluble in water, may have industrial and pharmaceutical applications. Analysis and hydrolysis showed that it consists of the calcium and magnesium salts of a polyuronide composed of D-glucuronic acid combined with D-galactose and L-arabinose. Gentle hydrolysis in acid solution liberates all of the L-arabinose and some of the D-galactose, and leaves a polyuronic acid combined with D-galactose. More drastic hydrolysis leaves a simple aldobionic acid composed of D-glucuronic acid combined with one molecule of D-galactose.

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Diethylaminoethyl Ester of Benzylpenicillin

I. Methods for the Determination of the Rate of Hydrolysis*,†

By H. L. DINSMORE‡ and S. D. BAILEY§

As a first approximation in establishing the therapeutic value of the diethylaminoethyl ester of benzylpenicillin, the rates of hydrolysis *in vitro* have been determined in buffered mediums covering the *p*H range from 2 to 8. The progress of hydrolysis has been followed in this study by removal of aliquot portions of the reacting solution and the successive determinations of the partition coefficients, using butanol as the other phase. From the known partition coefficients of the pure ester and benzylpenicillin, the amount of each component present has been determined, using ultraviolet spectroscopic assay of the partitioned layers. In the case of the hydriodide salt, a solubility method is reported which confirms the hydrolysis rates given by the partition coefficients.

AN IMPORTANT recent development in the use of penicillin against disease is the attempt to obtain more localized action in some specific tissue by modifying the carboxylic acid portion of the molecule. In addition to various salts that may be prepared, the use of esters offers a great variation in such physical properties as solubility and fat/water distribution. That the esterified penicillin will itself be inactive is no serious limitation if appreciable hydrolysis of the ester to yield free penicillin can confidently be expected to take place at the desired site in the body. It is likewise desirable that such hydrolysis be delayed until the ester arrives at the desired site. A method for the determination of the extent and rate of hydrolysis as a function of *p*H would therefore be of value. The present paper reports the results of this type of investigation[1] on both the hydrochloride and the hydriodide salts of the diethylaminoethyl ester of benzylpenicillin, hereafter referred to simply as "the ester."

EXPERIMENTAL

In following the hydrolysis of the ester quantitatively with time, observations are desired which are direct, requiring at most a few minutes each.[2] Because of these requirements, two simple physical measurements were selected for study: (*a*) the change in *solubility* with hydrolysis, and (*b*) the change in *partition coefficient* with hydrolysis, the results to be determined spectroscopically. These techniques will now be described in detail. All work was done at a constant room temperature of 25°.

The solubility method is peculiarly suited to the study of the iodide salt of the ester, which is only sparingly soluble in water, while any "free" penicillin, formed by hydrolysis, is highly soluble. Accordingly, if a saturated solution of the ester is maintained in equilibrium with excess undissolved ester, any appreciable increase in total penicillin solutes with time above the initial solubility of the ester iodide can be regarded as a direct result of hydrolysis to soluble free penicillin.

Solubility Method Procedure.—Approximately 0.2 Gm. of the ester iodide was dissolved in 10 cc. of 0.1 *M* phosphate buffer. The buffers employed were made by appropriate admixture and dilution of 0.2 *M* solutions of H_3PO_4, NaH_2PO_4, and Na_2HPO_4 to give *p*H values[3] of 2.2, 3.4, 4.9, 6.0, 6.7, and 7.4. After vigorous shaking for the first four minutes, the suspension was allowed to stand and was only occasionally shaken thereafter, as required to prevent all undissolved sample from settling out. After each desired time interval, a 1-cc. aliquot of clear solution was withdrawn and was transferred to suitable containers along with 5 cc. of blank buffer as diluent. On completion of the hydrolysis, all withdrawn portions were assayed for total dissolved benzylpenicillin (both free and as ester) by scanning the ultraviolet absorption near 263 mμ against an appropriate blank, using 1-cm. quartz cells and a Cary Model 11-M spectrophotometer. The difference in absorbance at 263 mμ (phenyl) and near 280 mμ (background) proved to be a reliable function of concentration.

Withdrawal of clear solution from the turbid suspension was accomplished simply by using a pipette with its tip wrapped in a filter-paper sheath, prepared several hours earlier by wrapping the tip with filter paper wet with alcohol, holding it tight with a small rubber band, and allowing the alcohol to evaporate in a warm oven. After withdrawing

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† The hydriodide salt of this ester is produced under the trademark "Neo-Penil" by the Smith, Kline and French Laboratories.

‡ Present address: Commercial Solvents Corp., Terre Haute, Ind.
§ Present address: Philadelphia Quartermaster Depot, Philadelphia, Pa.

[1] The authors wish to acknowledge the assistance of Miss Frances T. Lofton in certain phases of the experimental work.

[2] The unsuitability of time-consuming microbiological procedures (such as the agar plate assay) to closely timed studies is obvious.

[3] All *p*H measurements were made with glass electrode on a Beckman *p*H meter.

a little more than 1 cc. (slowly, in order to avoid foaming) the paper tip was removed, and the volume was adjusted to 1 cc. for transferral. This technique was used in all the work reported. A more elaborate and reliable technique is to filter 1.5-cc. aliquots (approximate) of the turbid suspension through sintered glass micro-funnels and to take 1 cc. of the (clear) filtrate for transferral.

The partition method, unlike the preceding one, appears to be of general application, and in the present study it was employed for both iodide and the chloride salts of the ester. The use of this method depends on a significant difference in partition coefficient K for the ester as contrasted with that for "free" penicillin. In the present work, the immiscible solvent pair employed was *n*-butyl alcohol/aqueous phosphate buffer. If the aqueous *p*H is 5 or above, the ester favors the organic phase whereas any free penicillin favors the aqueous phase. Partially hydrolyzed samples of the ester, accordingly, lead to observed values of partition coefficient K_s which are intermediate between the values K_e of the pure ester and K_a of a free penicillin salt such as potassium benzylpenicillin. From these three observables, the mole fraction X_e of unhydrolyzed ester, or X_a of its hydrolysis product, can be calculated by the following equation:[4]

$$X_e = 1 - X_a = \left[\frac{K_s - K_a}{K_e - K_a}\right]\left[\frac{K_e + 1}{K_s + 1}\right]$$

where K values refer to the ratio of concentrations in butanol to aqueous layers.

Partition Method Procedure.—Approximately 0.1 Gm. of the ester iodide or chloride was dissolved in 40 cc. of phosphate buffer, 0.1 *M* in work with the iodide, and 0.05 *M* in work with the chloride. After each desired time interval, a 5-cc. aliquot was transferred to a separatory funnel containing 5 cc. of butanol (Merck, reagent grade) and the layers were shaken together vigorously for two minutes. After separation, each layer was assayed immediately (without dilution) by ultraviolet absorption at 263 mμ (phenyl) as previously described. The same procedure was followed for control runs on potassium benzylpenicillin. Absorption near 320 mμ was also noted in connection with stability of the penicillin structure, since degradation products formed in weak acid give absorption at this wave length.

In order to obtain satisfactory partitions with buffers below *p*H 5, a slightly modified procedure was required, since with decrease in alkalinity, the K value of the ester rapidly decreases as its amine portion in acid becomes predominantly a water-soluble ammonium ion. A suitable difference of partition coefficients is restored simply by rebuffering each withdrawn 5-cc. aliquot to approximately *p*H 6 by the addition of a definite amount of 0.1 *M* NaOH and then partitioning with butanol; this NaOH solution can be added to the separatory funnel at the time of shaking and calculation can be made for the resulting unequal volumes of the two layers. Alternatively, one can start with an initial solution of the sample that is twice as concentrated, withdraw a 5-cc. aliquot, and after rebuffering it to *p*H 6, further dilute with *p*H 6 buffer to a volume of 10 cc. Of this 10 cc., only 5 cc. is subsequently taken for distribution with butanol. Both procedures were used in the present studies.

RESULTS AND DISCUSSION

Two sets of hydrolysis studies by the solubility method were carried out on the ester iodide at each of the six *p*H values previously listed. Aliquots were withdrawn for spectroscopic assay after five time intervals covering the range three to two hundred minutes. The data for the second set of runs are presented graphically in Fig. 1; the results for the first set are nearly identical except for scattered errors that were clearly occasioned by poor withdrawal technique in the earlier experiments. The percentage of hydrolysis has been computed from the increase in spectral absorbance above the extrapolated value at zero time. Incidentally this later value gives the solubility of the pure ester iodide to be about 1.25 Gm./100 cc. The graphs demonstrate that hydrolysis of the ester is quite rapid at *p*H 7.3 and decreases in rate progressively as the *p*H drops to 4.9. The rate again increases with further lowering of the *p*H; but below *p*H 3.4, actual degradation of the penicillin structure

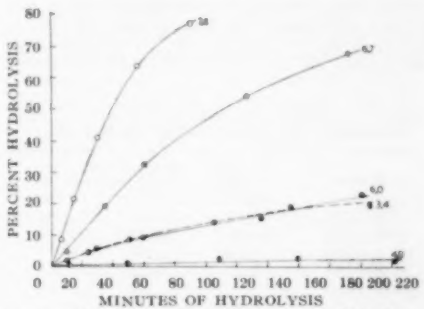

Fig. 1.—Hydrolysis of ester iodide in 0.10 *M* buffers, from solubility data.

becomes rapid, as shown by increases in foreign spectral absorption. Quantitative results, tabulated in Table I, show that in one hour the ester iodide is more than half hydrolyzed at *p*H 7.3, and is approximately one-fourth hydrolyzed at *p*H 6.7, but is less than 10% hydrolyzed at *p*H values from 6.0 to 3.4.

Apart from satisfactory reproducibility in the two series of solubility experiments, the correctness of the results will be supported later by their close check with the results obtained by the partition method. The average experimental error, which could scarcely be set at less than 10% because of the use of only 1-cc. pipettes, might be reduced appreciably by withdrawal of larger volumes.

[4] This formula was developed by Dr. R. H. Horowitz, of these laboratories, and H. L. Dinsmore, and assumes that the two components of the blend are distributed independently, as if each alone were present. Although a useful relationship for three independent components cannot be derived in terms of a few convenient observables, the binary formula remains useful in case of additional species which are related in a fixed (though undetermined) ratio to one or both of the two distinct components. Thus the presence of a weak acid in both unionized form and as an anion constitutes but one component when a fixed ratio of these is maintained, as in a particular buffer medium.

TABLE I.—RATE OF HYDROLYSIS OF DIETHYLAMINOETHYL ESTER OF BENZYLPENICILLIN

	Per Cent Hydrolysis of HI Salt						Per Cent Hydrolysis of HCl Salt		
	Solubility (1)		Solubility (2)		Partition			Partition	
pH of 0.1 *M* Buffer	%	Min.	%	Min.	%	Min.	pH of 0.05 *M* Buffer	%	Min.
7.4	25	16	25	20	25	17	7.4	25	16
	50	37	50	49	50	38		50	40
6.7	25	48	25	68	25	46	6.8	25	55
	50	94	50	160	50	110		50	136
6.0	10	100	10	110	10	64	6.1	5	210
	20	200	20	210	20	175		..	..
4.9	5	150	< 5	200	< 5	220	5.3	5	170
..	..	..	..	..	..	..	4.6	5	100
								10	210
3.4	10	130	10	165	10	64	3.5	10	62
	..	..	..	..	20	175		20	125
2.2	25	40	25	25	..	..	2.7	25	50

Hydrolysis studies by the partition method were carried out for both the ester iodide and chloride, in addition to analogous control runs on potassium benzylpenicillin. In each case, only one series of runs was made at each of the several *p*H values; observations were taken after five or six time intervals up to two hundred and sixty minutes. From the observed values of K_x and K_s and the extrapolated value for K_e (K_x at zero time) the per cent hydrolysis for each aliquot was calculated. The results are presented graphically in Figs. 2 and 3. The more important numerical data on K values are tabulated in Table II. Quantitative results on all the studies are compiled in Table I.

The tabulated and graphical partition results show, for both the ester iodide and chloride, essentially the same hydrolysis behavior with *p*H as has already been observed by solubility studies on the iodide: hydrolysis is least rapid from *p*H 5 to 6 and is pronounced above *p*H 7. Comparison of the results (in Table I) for the two alternative methods of investigation of the iodide shows good enough agreement to leave no doubt as to their validity, although closer precision would be desired. Undoubtedly the partition results are the more precise ones in view of the larger volumes dealt with. The great over-all similarity of results obtained in the partition studies on the two halide salts is not without some interesting differences, the iodide being least hydrolyzed near *p*H 5, whereas the chloride is least hydrolyzed near *p*H 6. The actual K_x values for the two halides (see Table II) show considerable diversity of behavior with *p*H. For the iodide, the values of K range from about 10 at *p*H 7.4 to 1.5 at *p*H 4.9. In contrast, for the chloride the range of K_x values is from about 60 at *p*H 7.4 to 1.1 at *p*H 5.3. These K_x values give an indication of the relative amounts of ester existing as free base at each *p*H.

These several sets of results on hydrolysis behavior with *p*H suggest that the entity particularly susceptible to hydrolysis is the free amine base of the ester, which naturally is present to greater extent in increasingly alkaline buffers. Hydrolysis occurs much less rapidly under conditions of acidity than under equivalent conditions of alkalinity, showing that the ammonium salt is very much more resistant than the free base. Since appreciable free amine still exists in weakly acid media, the minimum hydrolysis would not be expected at neutrality (*p*H 7) but more likely in the range *p*H 5 to 6, as actually observed. The increase of hydrolysis again in

TABLE II.—EFFECT OF *p*H ON PARTITION COEFFICIENTS IN BUTANOL/PHOSPHATE BUFFERS

Buffer[a] *p*H	Potassium Benzyl-Penicillin	Diethylaminoethyl Ester of Benzylpenicillin HCl Salt	HI Salt
7.5 (7.4)	0.77	ca. 60	ca. 10
6.8 (6.7)	0.68	ca. 20	7.70
6.1 (6.0)	0.64	3.30	4.05
5.3	0.79	1.22	..
4.9	..	..	1.55
4.6	0.86	0.79	..

[a] Values in parentheses are those used in work with the HI salt.

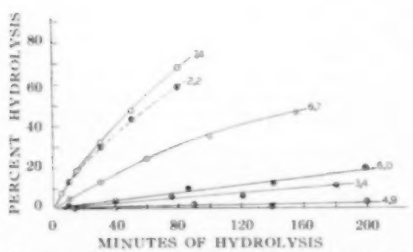

Fig. 2.—Hydrolysis of ester iodide in 0.10 *M* buffer, from partition data.

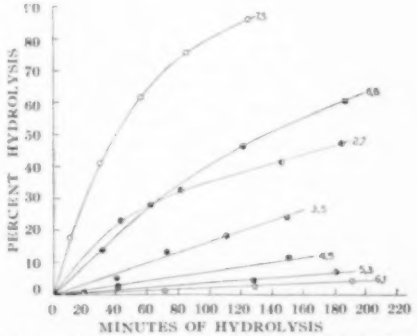

Fig. 3.—Hydrolysis of ester chloride in 0.5 *M* buffers, from partition data.

stronger acid media almost certainly involves a mechanism in which the ester ammonium ion is involved and undoubtedly is aided by degradation of penicillinic acid nearly as fast as it is formed.

Apart from furnishing values for K_2, the analogous partition and spectroscopic studies on potassium benzylpenicillin have enabled observation of the comparative stability of the penicillin structure under different conditions of *p*H and of chemical combination. Absence of spectral changes and constancy of K_2 values both show that potassium benzylpenicillin is not appreciably altered during three hours in buffers from *p*H 5.3 to 7.5. However, degradation occurs below *p*H 5, becoming quite rapid at *p*H 3.5 and almost instantaneous near *p*H 2. Not only is this indicated by changing values of the partition coefficient for successive aliquots but especially by definite spectral changes.

Under mildly acidic conditions (*p*H 3.5) a well-defined band at 320 mμ progressively increases for the first one hundred minutes, then starts to decrease while at the same time steadily increasing absorption toward shorter wave lengths begins near 260 mμ. In stronger acid, only the latter absorption is significant after the first few minutes, and it continues to increase rapidly. These observations confirm the already known fact that the band at 320 mμ is due to an intermediate[5] rather than to the ultimate acid-degradation product. The partition studies show the interesting fact that the component leading to 320 mμ absorption goes predominantly into the butanol layer, whereas the product that absorbs below 260 mμ is overwhelmingly water-soluble.

Possibly the most significant spectroscopic contribution of the present studies is the observation that there is drastically less degradation of penicillin structure in acid media for the ester than for potassium benzylpenicillin. Thus at *p*H 3.5 the amount of decomposition shown by potassium benzylpenicillin in thirty minutes is much more than that shown by the ester after three hours; and even near *p*H 2 the degradation of the ester after one hour is not as great as that of free penicillin three minutes after solution. This evidence makes it reasonable to conclude that the ester resists such degradation entirely, the latter occurring only with such free penicillin as has been formed by hydrolysis. That this explanation is consistent may be attributed to the instability characteristic of the penicillinic acid molecule under these conditions. Naturally the penicillinic acid molecule predominates over the penicillin anion as acidity increases—a fact reflected in the steady increase in K_a values for potassium benzylpenicillin as the *p*H of the buffered solutions decreases below *p*H 6.

Before concluding the discussion, it is appropriate to point out a limitation to high accuracy in the present studies; namely, failure of the buffered solutions of the ester to remain at a constant *p*H during a given set of observations. Measurements on 0.2% solutions of the ester chloride in approximately 0.03 *M* buffers initially at *p*H 7.5 and 6.6 showed gradual decrease in *p*H to values of approximately 7.1 and 6.4 in two hours, corresponding to about 75% and 20% hydrolysis, respectively. This falling-off was probably less rapid in subsequent work with 0.1 *M* buffers.

[5] Penicillinic acid (as an oxazolone) is unstable in acid but is somewhat stable in nonionizing media (1, 2).

SUMMARY

The present investigations show that hydrolysis of the HCl and HI salts of the diethylaminoethyl ester of benzylpenicillin occurs rapidly in either weakly alkaline or strongly acid dilute solutions, with maximum stability near *p*H 5 to 6. The progress of hydrolysis can be followed closely, simply by successive determinations of the partition coefficient, with butanol as the other phase, using ultraviolet spectroscopic assay of the layers. This method appears to be of general application. An independent series of studies has also been made on the HI salt by a solubility method which is probably unique to this particular case, giving results in fair agreement with those obtained by partition measurements and confirming their validity.

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Diethylaminoethyl Ester of Benzylpenicillin

II. Rate of Hydrolysis in a Pharmaceutical Suspension*,†

By ROBERT E. KELLER‡

The hydrolysis rate of the diethylaminoethyl ester of benzylpenicillin in a pharmaceutical suspension has been determined by a single contact partition method. The partition coefficients K_e of the pure ester, K_x of the partially hydrolyzed sample, and K_p of sodium penicillin G are calculated from ultraviolet absorption data. From these three experimental partition coefficients, the per cent of ester hydrolyzed at any interval is calculated by a suitable equation. Hydrolysis rate curves, representing 5°, 25°, and 37° studies, are plotted over a period of 168 hours.

RECENTLY considerable interest has been evinced in certain esterified penicillins that localize in some specific body tissue. In the ester form, these penicillins possess no antibiotic activity and it is only when they undergo hydrolysis that they yield active penicillin. Therefore, their therapeutic action depends upon the release of active penicillin within the body. It is assumed that the active penicillin would be present as a soluble salt of penicillin "G," presumably as sodium penicillin "G." The selective action is dependent upon a preferential localization of the inactive ester in some specific tissue.

Since the dosage form contains the inactive ester, it is necessary to determine the rate of hydrolysis in reconstituted commercial preparations. This information is of value in determining the full dosage of the intact ester after it has been stored at various conditions of temperature. This paper reports the results of this type of investigation as well as the analytical method employed. The material under study is "Neo-Penil"[1] (hereafter referred to as "the ester") as prepared in a pharmaceutical suspension.

EXPERIMENTAL

The analytical method employed is based upon a change in the partition coefficient of the partially hydrolyzed material. The technique of liquid-liquid extraction (more specifically, the partition of substances between pairs of immiscible solvents) is a very convenient tool for analysis of a great diversity of compounds (1). The single contact extraction procedure employed in this investigation is similar to a method described in Part I of this paper. It depends upon a difference in partition coefficient K for the ester as contrasted with that for sodium penicillin "G."

With a chloroform and aqueous phosphate buffer pair, the ester favored the organic phase, whereas sodium penicillin "G" favored the aqueous phase. Partially hydrolyzed samples of the ester produced an intermediate coefficient K_x. The partition coefficient K_e of the pure ester and K_p of the sodium penicillin "G" were obtained from freshly prepared solutions. From these three experimental values the per cent of ester hydrolyzed at any predetermined interval was calculated by the equation shown below:

$$\% \text{ Ester Hydrolyzed} = \left[1 - \left(\frac{K_x - K_p}{K_e - K_p}\right)\left(\frac{K_e + 1}{K_x + 1}\right)\right] 100$$

$$K = \frac{\text{Chloroform phase (O. D. 264 m}\mu - \text{O. D. 280 m}\mu)}{\text{Aqueous buffer phase (O. D. 264 m}\mu - \text{O. D. 280 m}\mu)}$$

$K_x = K_{\text{SKF 1114-J Formulation in a Buffered Suspension}}$

$K_e = K_{\text{Hydriodide Salt of Diethylaminoethyl Ester of Benzyl penicillin}}$

$K_p = K_{\text{Sodium Penicillin G}}$

The temperatures 5°, 25°, and 37° were used to simulate the actual extreme conditions under which an ester suspension might be allowed to remain after it has been reconstituted. Eighteen formulations were transferred from their commercial rubber-stoppered vials to short weighing bottles. After being mixed with water, the suspensions were allowed to remain at the various temperatures. The intervals at which the reconstituted samples were analyzed extended over a period of 168 hours.

PROCEDURE

Approximately 0.5 Gm. of the ester formulation which contains a citrate buffer is reconstituted with 1.5 cc. of distilled water. The sample is swirled by an electric rotating apparatus at a speed of 140 r. p. m. for five minutes to suspend the formulation in the diluent. After the desired time interval, the suspension is again mixed for five minutes, transferred to a 250-cc. volumetric flask with *p*H 6.0, 0.2 *M* phosphate buffer, and dissolved. The dissolved suspension is made up to volume with additional buffer solution and a 15-cc. aliquot is pipetted into a 60-cc. glass stoppered bottle.

* Received June 6, 1952, from the Research and Development Division, Smith, Kline and French Laboratories, Philadelphia 1, Pa.

† Presented to the Medicinal Chemistry Division of the American Chemical Society, Milwaukee meeting, April 1, 1952.

‡ The author wishes to thank W. C. Ellenbogen for his many helpful discussions.

[1] Smith, Kline and French trademark for the hydriodide salt of diethylaminoethyl ester of benzylpenicillin.

Fifteen cubic centimeters of reagent grade chloroform is added and the two phases are mixed vigorously with a vertical reciprocating shaker for five minutes. The phases are separated with a separatory funnel, and exactly 10 cc. of the buffer phase is mixed with 1 cc. of buffer reagent. In a separate flask, 10 cc. of the chloroform phase is mixed with 1 cc. of chloroform reagent. The resulting solutions are assayed for total dissolved benzylpenicillin, i. e., both "free" and as ester by scanning the ultraviolet region from 300 to 250 mμ against an appropriate blank, using 1-cm. quartz cells and a Cary Model 11-M recording spectrophotometer. The difference in optical density of the phenyl group absorption at 264 mμ and the background absorption at 280 mμ is used as a function of concentration. The same procedure used for the formulations is followed with sodium penicillin G and the hydriodide salt of diethylaminoethyl ester of benzylpenicillin.

RESULTS

The partition coefficient and per cent hydrolysis data are tabulated in Table I. There is an apparent increase in hydrolysis with a reversal effect over the first six hours for the 25° and 5° studies. Actually, the 5° study decreases in per cent hydrolysis to the seventy-two hour interval.

Table II shows the *p*H drift of the suspension as measured with a Beckman Model G *p*H meter. It is to be noted that at *p*H 6.0, the time interval corresponds to the one where the reversal effect ceases with the 25° and 5° studies. It is suggested the initial abnormal peaks shown by the hydrolysis data are related to the *p*H drift of the suspension, since they revert back to the normal curve pattern at that time interval where the *p*H of the suspension is 6.0 and the hydrolysis rate a minimum. This indicates that in applying distribution law theory to this problem, one is concerned not just with two entities, but with the fact that over a certain narrow *p*H range, as a result of ionization or degradation effects or both, other species are present which are soluble in chloroform and contain the phenyl radical.

This establishes the fact that the method used to calculate per cent hydrolysis is in error in this region. The magnitude of this error appears to be roughly the same at each of the temperatures employed, which means its effect on the calculated per cent hydrolysis is slight at the highest temperature, but large at the lowest temperature.

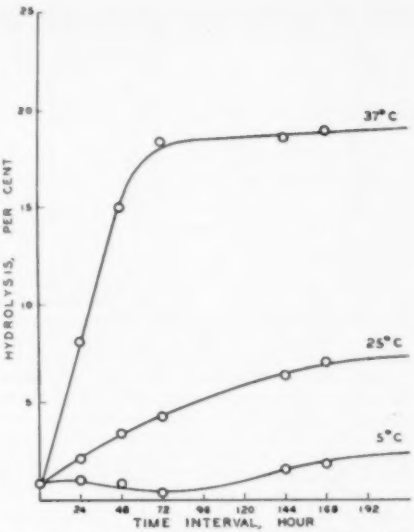

Fig. 1.—Hydrolysis curves for reconstituted ester.

As indicated in Fig. 1, the rate of hydrolysis at 37° is quite rapid up to the seventy-two hour interval, where it levels off and for all practical purposes remains constant at 19%. At 25°, the hydrolysis assumes a progressive rate throughout the week and more nearly resembles a straight line with a maximum of 7% at 168 hours. At 5°, the curve actually decreases to seventy-two hours, at which time a steady increase is indicated with a maximum of 2%. The previously mentioned initial peaks over the first six hours have been deleted from the curves, due to their insignificance in relation to the normal curve pattern and to the original purpose of the investigation.

In the present work, the immiscible solvent pair, chloroform and aqueous phosphate buffer, was chosen due to its selective action on the constituents to be partitioned. The greater selectivity that can be introduced by the solvent system, the greater the accuracy of the assay method. A buffer of *p*H 6.0 was selected, since it has been found by Dinsmore and Bailey (2) that the hydrolysis rate of the

TABLE I.—HYDROLYSIS DATA ON ESTER SUSPENSIONS

Time Interval, Hr.	5° C.		25° C.		37° C.	
	K_x[a]	%[b]	K_x	%	K_x	%
1/12	15.833	0.85	15.833	0.85	15.833	0.85
1	13.500	1.90	14.472	1.43	12.780	2.30
2	13.701	1.80	12.540	2.44	12.600	2.40
3	13.492	1.90	10.457	3.90	11.439	3.20
6	14.296	1.51	13.068	2.13	10.793	3.49
24	15.314	1.05	12.354	2.55	6.952	8.15
48	15.917	0.82	11.091	3.41	4.302	15.08
72	17.044	0.41	9.986	4.34	3.554	18.49
144	14.037	1.63	8.091	6.44	3.519	18.68
168	13.414	1.78	7.591	7.12	3.450	19.05

[a] $K_x = 18.330$, $K_p = 0.042$.
[b] Per cent of original sample hydrolyzed.

TABLE II.—pH DRIFT OF RECONSTITUTED SAMPLE

Time Interval, Hr.	5° C.	25° C.	37° C.
0	6.91	6.91	6.91
5	6.55	6.14	....
24	6.43	5.89	....
48	6.28	5.66	....
72	6.08	5.51	4.79
120	6.01	5.30	4.70
144	5.97	5.29	4.62
168	5.97	5.27	4.57

ester is at a minimum between 5 and 6 and increases in either pH direction. Therefore, the possibility of further hydrolysis in the buffer phase after partition was reduced to a minimum. In addition, it was necessary to consider a pH where penicillin is quite stable. At the lower pH values, penicillin degradation causes spectral changes and an alteration of the characteristic absorption curve.

The experimental procedure requires a total analysis time of one hour for each time interval. One of the advantages of the assay method is the complete absence of weighed samples, as the distribution ratio is independent of the sample size. The reproducibility is ±0.5%.

SUPPLEMENTARY ASSAYS

The present work was preceded by several solubility studies that have since served to verify the partition data. The ester is sparingly soluble in the small volume of water used for reconstitution. An increase in solubility is credited to the hydrolysis reaction which serves effectively to eliminate the intact dissolved ester in the diluent. This process allows more ester to be dissolved and taken into solution by the suspending medium. Therefore, a measurement of the penicillin or iodide ion concentration in the filtrate at any interval is a direct determination of increasing solubility as well as an indirect estimate of the hydrolysis rate.

A series of ester formulations was reconstituted and the filtrates assayed at selected intervals over a 168-hour period at 25°. Two assay methods were used in the experimental work. The first was an ultraviolet absorption procedure based (as in the partition work) on the characteristic absorption of the phenyl group of benzylpenicillin. Two grams of the ester formulation was suspended in 6.0 cc. of distilled water. Three cubic centimeters of the filtrate was diluted volumetrically to 50 cc., and this solution was read at 264 mμ with a Beckman Model DU spectrophotometer.

With the second method, the iodide ion concentration was measured by a potentiometric titration procedure. Twenty-five cubic centimeters of the volumetric solution prepared for the absorption study was pipetted into a 250-cc. beaker. One cubic centimeter of 0.2 *N* nitric acid was added, followed by 100 cc. of water, and the mixture was stirred by a magnetic stirrer while being titrated with 0.015 *N* silver nitrate solution. The titration curve was followed with a Beckman Model G pH meter with a silver-mercury, mercurous sulfate electrode pair.

The data with these two analytical solubility methods have agreed within ±0.1% throughout the pH range where penicillin is stable. The ultraviolet method, however, was not valid for a longer time interval than twenty-four hours, since the pH drop produced a small amount of unstable penicillin which altered the characteristic absorption curve.

With the potentiometric procedure, it was conceivable that hydriodide could ionize into the diluent from the solid suspended ester molecule and leave the ester in the suspension. Thus, the iodide ion concentration would not necessarily be a measure of ester solubility. Consequently, neither of the two solubility procedures gave conclusive hydrolysis rates, and it was necessary to use the partition method described in this paper as a final solution to the problem. It has been found that the rates of hydrolysis from the solubility studies parallel those from the partition work with a constant deviation of ±0.4 to 0.8%.

SUMMARY

The hydrolysis rate of the diethylaminoethyl ester of benzylpenicillin in a pharmaceutical suspension has been determined by a single contact partition method. The method depends upon a difference in partition coefficient K for the ester as contrasted with that for one of the hydrolysis products, sodium penicillin G. The immiscible solvent pair is chloroform/aqueous phosphate buffer.

After partition, each immiscible solvent phase is assayed for benzylpenicillin (either "free" or ester or both) by scanning the ultraviolet region from 300 to 250 mμ with a Cary Model 11-M recording spectrophotometer. The difference in optical density of 264 mμ (phenyl group) and 280 mμ (background) is used as a function of concentration.

The partition coefficients K_e, of the pure ester, and K_p, of sodium penicillin G, are obtained from freshly prepared solutions. Partially hydrolyzed samples of the ester produce an intermediate coefficient K_x. From these three partition coefficients, the per cent of ester hydrolyzed at any interval is calculated by a suitable equation.

Hydrolysis rate curves are plotted over a 168-hour period. The maximum hydrolysis is 19 per cent, 7 per cent, and 2 per cent at 37°, 25°, and 5°, respectively.

One of the advantages of the assay method is the complete absence of weighed samples. The reproducibility of the method is ±0.5 per cent.

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Tolerance of Rats Toward Amphetamine and Methamphetamine*

By JOSEPH W. E. HARRISSON, CLARA M. AMBRUS, and JULIAN L. AMBRUS†,‡

It has been shown that the body temperature-increasing effect of *d*-amphetamine as well as *d*-desoxyephedrine (methamphetamine) is less after repeated daily injections. No significant difference was noted between the reactions toward these two drugs.

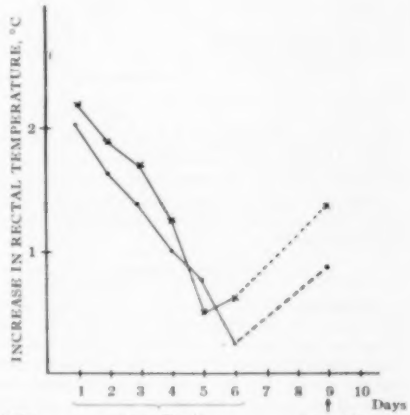
CONSIDERABLE DEBATE appears in the earlier literature as to whether there is a physical tolerance or even a dependence developed toward amphetamine or only habituation on a psychological basis. Guttman (1), Gorrell (2), Davies and Stewart (3), Korns and Randall (4), Ulrich (5), and Bloomberg (6) did not observe any tolerance in patients. Lesses and Myerson (7) and Rosenberg (8) found that, in obesity cases, the dose had to be increased in order to maintain the effect during prolonged administration. The same authors also observed a decreased pressor effect of the drug after a few days' administration. Similar findings as to blood pressure effect were reported by Hill (9) and Reifenstein and Davidoff (10, 11). The tachyphylactogenic effect of amphetamine and its derivatives in experimental animals regarding blood pressure effect was described by Tainter, *et al.* (12–14), Pinkston, *et al.* (15, 16), and Winder, *et al.* (17). Similar phenomena were observed on the blood pressure of man by Carl and Turner (18); Searle and Brown (19) reported that 3 mg./Kg. of amphetamine administered to rats ten times within forty days did not result in any decrease of the spontaneous motility stimulating effect. Ehrich and Krumbhaar (20), on the other hand, found that in rats the lethal dose of amphetamine is increased after several injections of sublethal doses (30–400 mg./Kg.). They observed subjectively that the excitatory effect decreased, following prolonged administration. The doses given by Ehrich and Krumbhaar (20) appear to be high, and those of Searle and Brown (19) given too infrequently, to be able to draw practical conclusions.

The relative stimulating effect of *d*-methamphetamine (*d*-desoxyephedrine), compared with that produced by amphetamine, was investigated by Novelli and Tainter (14) who concluded it was more effective. At about the same time, Ivy and Krasno (24) reviewed all available literature on this topic because of the reported use of methamphetamine by the German Army to produce stimulation without unwanted side effects. They concluded that it might be more potent than *d*-amphetamine but found no evidence of lack of side reactions.

In this study, it was attempted to develop data on the question of tolerance in rats toward amphetamine and to compare its effect with that of desoxyephedrine. The body temperature-increasing effect of these compounds [Beyer (21), Delaunois and Marri (22), and Merdinger (23)] was used as a convenient and objective test for the developing resistance of the organism.

METHODS

Female albino rats of 180–200 Gm. body weight were used for these experiments. *d*-Amphetamine sulfate and *d*-desoxyephedrine hydrochloride (methamphetamine hydrochloride) were dissolved in normal saline. All doses were calculated to the base. Injections were given subcutaneously. Rectal temperature was taken by mercury thermometers, before administration of the drugs and one hour



DOSE: 5 mg./Kg./day 5 mg./Kg.

S. E. Amphetamine:
0.21 0.13 0.12 0.09 0.10 0.23 0.13

S. E. Desoxyephedrine:
0.21 0.17 0.16 0.14 0.09 0.11 0.13

•———• Amphetamine (N = 15)
x———x Desoxyephedrine (N = 14)

Fig. 1.—Mean increases of rectal temperatures.

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† The authors wish to acknowledge the assistance of George V. Rossi.
‡ Aided in part by a grant from Eli Lilly and Co.

TABLE I.—AMPHETAMINE, 5 MG./KG.

No.	Body Wt., Gm.	1st Day Temp. before Inj.	1st Day Temp. 1 Hr. after Inj.	1st Day Difference	2nd Day Temp. before Inj.	2nd Day Temp. 1 Hr. after Inj.	2nd Day Difference	3rd Day Temp. before Inj.	3rd Day Temp. 1 Hr. after Inj.	3rd Day Difference	4th Day Temp. before Inj.	4th Day Temp. 1 Hr. after Inj.	4th Day Difference
1.	182	36.8	38.7	1.9	37	38.7	1.7	37	38.7	1.7	37	37.9	0.9
2.	180	37.1	39	1.9	37.2	39	1.8	37.1	38.4	1.3	37	38.1	1.1
3.	195	36.2	38.6	2.4	36.5	37.7	1.2	37	38	1.0	36.9	37.9	1.0
4.	179	36.8	39.7	2.9	36.9	38.8	1.9	36.9	38.7	1.8	36.5	37.8	1.3
5.	190	36.4	39.4	3.0	36	36.6	0.6	36.5	37.1	0.6	36.8	37	0.2
6.	190	37	39.1	2.1	37.4	38.2	0.8	37.2	38.1	0.9	37.2	37.8	0.6
7.	192	37.2	38.6	1.4	37.2	38.1	0.9	37.2	38.3	1.1	37.2	38.5	1.3
8.	186	37.4	39	1.6	37.4	38.8	1.4	37.5	38.8	1.3	37.2	38.2	1.0
9.	185	37.3	37.9	0.6	37.4	39.3	1.9	37.3	39	1.7	37.6	38.9	1.3
10.	190	36.8	39.4	2.6	37.6	38.8	1.2	37.2	39.1	1.9	36.8	38.2	1.4
11.	200	37	39.3	2.3	36.9	38.6	1.7	37.3	38.2	0.9	36.9	38.1	1.2
12.	198	37.2	39.2	2.0	37.1	39.7	2.6	37.2	39.6	2.4	36.9	38.2	1.3
13.	200	36	39.1	3.1	37	38.6	1.6	37	38.2	1.2	37	38.4	1.4
14.	195	36.9	39.4	2.5	37	38.4	1.4	37.2	38.7	1.5	37.4	38	0.6
15.	182	37.2	37.3	0.1	37.3	39.2	1.9	37.2	38.9	1.7	37.3	38.2	0.9
Total of difference				30.4			22.6			21.0			15.5
Mean difference				2.02			1.50			1.4			1.03

No.	Body Wt., Gm.	5th Day Temp. before Inj.	5th Day Temp. 1 Hr. after Inj.	5th Day Difference	6th Day Temp. before Inj.	6th Day Temp. 1 Hr. after Inj.	6th Day Difference	Body Wt., Gm.	9th Day Temp. before Inj.	9th Day Temp. 1 Hr. after Inj.	9th Day Difference
1.	189	36.5	36.7	0.2	36.8	36.8	0.0	185	37.2	38	0.8
2.	178	36.8	37.2	0.4	36.8	37.4	0.6	180	37	37.7	0.7
3.	198	37	38.3	1.3	37.2	37.6	0.4	200	37	37.9	0.9
4.	183	36.2	37.3	1.1	37	37.2	0.2	197	37.3	38.3	1.0
5.	185	36.5	37	0.5	36.8	37.5	0.7	195	37.2	38.7	1.5
6.	188	37	37.6	0.6	37.1	37.4	0.3	194	37.5	38.7	1.2
7.	194	37.4	38	0.6	37.3	37.3	0.0	190	36.9	37.5	0.6
8.	186	36.8	37.2	0.4	37.2	37.4	0.2	195	37	37.9	0.9
9.	190	37.4	38.4	1.0	37.5	38.1	0.6	190	37.1	38.4	1.3
10.	195	37.2	38.2	1.0	37.5	38.3	0.8	192	37.3	37.8	0.5
11.	195	36.9	37.8	0.9	36.6	36.9	0.3	187	36.8	37.5	0.5
12.	200	37.1	37.8	0.7	37.2	37.2	0.0	195	36.9	37.7	0.8
13.	205	37.3	38.4	1.4	37	37.2	0.2	195	36.6	37.1	0.5
14.	200	37	37	0.0	37	37	0.0	200	37.2	38.4	1.2
15.	190	36.9	37.2	0.3	37.2	37.2	0.0	184	37	37.7	0.7
Totals of difference				10.4			4.3				13.1
Mean difference				0.69			0.29				0.89

after. Preliminary experiments indicated that the peak of temperature increase is reached at about one hour following the doses used. Injections were usually given and temperatures taken in the evening. Food was removed from the cages eight to ten hours previously.

OBSERVATIONS

During six consecutive days, 5 mg./Kg. of amphetamine or desoxyephedrine was injected daily, using two groups of 15 rats each. After the sixth day, administration of the drugs was discontinued for two days. On the ninth day, the usual doses were given again. The main increases of rectal temperatures with their standard errors are shown in Fig. 1 and Tables I and II. The body temperature-increasing effect of *d*-amphetamine as well as desoxyephedrine decreases during the six days of administration and increases again after a temporary discontinuation of the injections. There is no significant difference in the response to either of these two drugs. During the experimental period, there was no evidence of decrease of body weight.

SUMMARY

1. Amphetamine as well as desoxyephedrine increases the body temperature of rats.

2. This effect gradually decreases if the drugs are administered daily for six days.

3. Discontinuing the injections for two days results in an increase of the body-temperature effect.

4. There is no difference evidenced from this point of view between amphetamine and desoxyephedrine.

5. Literature on the tolerance and habituation toward this group of drugs is briefly reviewed.

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TABLE II.—DESOXYEPHEDRINE, 5 MG./KG.

		1st Day			2nd Day			3rd Day			4th Day		
No.	Body Wt., Gm.	Temp. before Inj.	Temp. 1 Hr. after Inj.	Difference	Temp. before Inj.	Temp. 1 Hr. after Inj.	Difference	Temp. before Inj.	Temp. 1 Hr. after Inj.	Difference	Temp. before Inj.	Temp. 1 Hr. after Inj.	Difference
16.	197	36.4	39.3	2.9	36.2	38.7	2.5	36.4	38.9	2.5	36.6	38.4	1.8
17.	190	37	38.9	1.9	36.8	38.7	1.9	36.8	38.2	1.4	37	38.2	1.2
18.	190	36.8	37.7	0.9	37	38.4	1.4	37.1	38.1	1.0	37.2	37.8	0.6
19.	195	37.5	40.1	2.6	37.2	39.2	2.0	37.2	39.2	2.0	37	38.8	1.8
20.	198	36.9	39.7	2.8	37.1	39.4	2.3	37.2	39.3	2.1	36.9	38.6	1.7
21.	200	37.1	39.8	2.7	37.3	39.2	1.9	37	38.7	1.7	37	38.2	1.2
22.	204	37.2	38.5	1.3	37	38.8	1.8	37.2	38.2	1.0	37.2	37.5	0.3
23.	202	37.2	38.8	1.6	36.9	38	1.1	36.8	38.4	1.6	36.9	37.6	0.7
24.	185	37.3	37.9	0.6	36.8	37.7	0.9	36.8	37.7	0.9	36.9	37.1	0.2
25.	180	37	38.9	1.9	37	37.7	0.7	37.2	39.1	1.9	37	38.6	1.6
26.	..	..	..	..	..	..	..	..	..	..	..	..	..
27.	197	36.5	39.3	2.8	36.7	39.5	2.8	36.9	39.4	2.5	37.3	39.3	2.0
28.	190	36	38.9	2.9	36.2	38.1	1.9	36.8	39	2.2	37	38.9	1.9
29.	189	37	39.7	2.7	37	39.5	2.5	37.1	39.1	2.0	36.8	38.3	1.5
30.	196	36.8	39.4	2.6	37.2	39.7	2.5	37	38.8	1.8	36.6	38.2	1.6
Totals of difference				30.2			26.2			24.6			18.1
Mean difference				2.16			1.87			1.76			1.29

		5th Day			6th Day				9th Day		
No.	Body Wt., Gm.	Temp. before Inj.	Temp. 1 Hr. after Inj.	Difference	Temp. before Inj.	Temp. 1 Hr. after Inj.	Difference	Body Wt., Gm.	Temp. before Inj.	Temp. 1 Hr. after Inj.	Difference
16.	195	36.8	37.7	0.9	37	37.8	0.8	190	36.8	38.4	1.6
17.	195	37.2	37.6	0.4	37	37.4	0.4	195	37.1	38.5	1.4
18.	195	38	38.5	0.5	37.3	37.9	0.6	195	37.3	39.1	1.8
19.	200	36.9	38	1.1	37.2	38.3	1.1	204	36.9	39	2.1
20.	190	37.3	38.1	0.8	37	38.1	1.1	185	36.5	39.1	1.6
21.	192	37.2	37.8	0.6	37.4	38	0.6	188	37.2	37.7	0.5
22.	198	37	37.2	0.2	37.3	37.3	0.0	190	37.1	38	0.9
23.	200	36.9	36.9	0.0	37.1	37.1	0.0	195	37	37.8	0.8
24.	185	37.3	37.3	0.0	37	37	0.0	187	37	37.9	0.9
25.	188	37.2	38.3	1.1	37	37.5	0.5	180	36.8	38.2	1.4
26.	..	..	..	..	..	..	..	..	..	..	..
27.	190	37	37.7	0.7	36.8	38	1.2	175	36.9	39.2	2.3
28.	185	37.2	37.7	0.5	36.9	37.6	0.7	180	37.2	39.1	1.9
29.	190	36.9	37.3	0.4	37.1	37.5	0.4	187	37.3	38.6	1.3
30.	200	37	37.2	0.2	37.3	38.1	0.8	196	36.8	38	1.2
Totals of difference				7.4			8.2				19.7
Mean difference				0.53			0.58				1.40

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The Effect of Wool Fat and Some of Its Constituents on the Absorption of Phenolsulfonphthalein through the Intact Skin*,†

By VISHNU N. BHATIA‡ and LOUIS C. ZOPF§

THE PROBLEM of absorption of drugs through the skin is not new. It has been known for a long time that mercury could be absorbed in toxic amounts if it was rubbed on the soles of the feet. As early as 1809, Massey (1) reported that drugs applied to the skin were absorbed and could be detected in the urine. It is now generally agreed that skin is not the impenetrable barrier it was once believed to be; but diversity of opinion remains, both as to the mechanics of absorption and as to relative merits of the various substances used as ointment bases.

Sulzberger, in discussing the work of Duemling (2), stated that "dermatologists will have to be more precise about what is to be accomplished by medication when applied to the skin."

The importance of absorption becomes even more evident when the work of Stakosch (3), who reported fatalities from the external application of sulphur, is considered. Sannicandro (4) made similar claims about the external application of salicylic acid. Neuroth and Lee (5) have stated that mercurials and phenolic derivatives may be dangerous when incorporated into bases containing relatively large quantities of water.

WOOL FAT

Wool fat is the purified, anhydrous, fatlike substance from the wool of sheep, *Ovis aries* (6). Its composition is not clearly defined (7, 8), but in general it contains the compounds listed in Table I.

The use of wool fat and cholesterol to enhance absorption has been much debated, and many contradicting views have been expressed on this subject. Among the earlier contributions on the effect of wool fat on absorption was the work of Luff (9) who stated that it was inferior to petrolatum. Saurland (10) agreed with this view. However, it has been stated that the presence of derivatives of cholesterol increases absorption (11); Bhatia and Zopf (12) also observed that a base containing wool fat caused greater absorption than one which was devoid of it. Breslauer (13) demonstrated that lanolin was among the best bases for antiseptics. His method consisted of studying the growth of organisms on substances which had been exposed to different antiseptic ointments.

Starkenstein and Hendrych (14) attributed to cholesterol a permeability-regulating action and concluded that it must exercise a protective action, since an insulin ointment which failed to produce any blood sugar lowering when rubbed on the normal skin of the rabbit produced a significant drop in the blood sugar level when the skin had previously been washed with petroleum ether, a solvent of cholesterol.

Eller and Wolff (15) also reported wool fat to be superior to petrolatum. Macht (16) found that wool fat was superior to other fats.

Thus it is evident that further investigation of the effect on absorption of wool fat, cholesterol, and allied substances would be of value, since there is a great deal of divergence of opinion and the data available are of a conflicting nature. The problem assumes greater importance when it is considered that cholesterol is a natural constituent of the skin and the cholesteryl esters are natural constituents of the greasy covering of the skin.

Various methods have been described for measuring absorption. Luff (9) measured penetration through a sheep's bladder suspended in a bath; Mischer (17) used histological techniques; Brown and Scott (18), in studying the absorption of methyl salicylate, used the combined area of both hands as the site of application for one hour, with the subject himself carrying out the massage.

The most suitable method seems to be that of Macht (16), consisting of applying test ointments to the shaved skin of animals and then detecting the absorbed material in the urine of the animals.

EXPERIMENTAL

Wool fat was saponified by the method of Powers, Leask, and Warner (19), and the following fractions were obtained: cholesterol, isocholesterol, aliphatic alcohols, and residue.

The following esters were synthesized by the method of Page and Rudy (20): cholesteryl stearate, cholesteryl palmitate, and cholesteryl acetate.

ABSORPTION STUDIES

Materials.—The absorption studies were carried out on fifty 150-Gm. albino rats, which were divided into 10 groups of 5 animals each. Petrolatum U. S. P. and polyethylene glycol ointment U. S. P. XIV were used as the two ointment bases, the former representing the oleaginous type of bases and the latter the hydrophilic type. Phenolsulphonphthalein in a concentration of 5% was

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TABLE I

Sterols	Terpene Alcohols	Acids	Aliphatic Alcohols
Cholesterol (approximately 15–20% of the total wool fat)	Lanosterol	Carnaubic	Cetyl
Dehydrocholesterol	Agnosterol	Myristic	Stearyl
Dihydrocholesterol	(Together they are known as "isocholesterol")	Palmitic	Ceryl
m-Cholesterol		Cerotic	Lanyl
Oxycholesterol		Lanopalmitic	Lanooctadyl
			Unsaturated alcohols with 10, 11, and 12 carbon atoms (40 or more)

used as the tracer, as this dye is rapidly excreted unchanged in the urine and is readily detected.

The following substances were incorporated in concentrations of 5% and 10% in the ointment of phenolsulfonphthalein prepared with each of the two bases: wool fat, cholesterol, isocholesterol, aliphatic alcohols from wool fat, residue from wool fat, cholesteryl stearate, cholesteryl palmitate, and cholesteryl acetate.

Method.—Each substance was tested on 2 groups of animals. The animals were anesthetized with ether, and a well-defined area (2 in. by 1.5 in.), extending on both sides of the spinal column between the forelegs and the tail, was clipped with a pair of animal clippers so as to remove the hair without damaging the epidermis. One gram of the ointment was applied evenly to this exposed area. The treated rats were wrapped with adhesive tape in such a manner that they could neither rub off (by contact with the cage) nor ingest any of the ointment. After being taped, the animals were placed in separate metabolism cages, and their urine was collected over a period of twenty-four hours. At the end of the period, they were again anesthetized and the tape was removed. The animals were then allowed to rest for one week before being used for further tests, as it was determined that it took from three to four days for their urine to be free of the absorbed dye.

The urine specimens thus collected were diluted to one liter and centrifuged. A solution of sodium hydroxide (5%) was added drop by drop to the diluted urine samples until the maximum pink color was developed. The intensity of the pink color produced by the interaction of the alkali and the dye was measured on a photoelectric colorimeter which had previously been calibrated by using dilutions of standard solutions of phenolsulfonphthalein.

In order to insure the accuracy of the colorimeter readings, urine from untreated rats was similarly diluted, treated with alkali, and examined in the colorimeter. No evidence of pink color was found.

RESULTS

The results obtained are shown in Table II. All values are stated in milligrams.

The abbreviations PET, P. E. G. O., and W. F. refer to petrolatum, polyethylene glycol ointment, and wool fat, respectively.

The results were statistically analyzed and "*t*" values for different combinations were calculated to ascertain if the values obtained were significantly different.

DISCUSSION

Wool Fat and Its Fractions.—This study establishes the fact that wool fat and the four fractions tested enhance the systemic absorption of phenolsulfonphthalein from both bases.

The increase in absorption caused by the residue is relatively little even though it is statistically significant.

Aliphatic alcohols show a relatively greater effect than the residue. However, this effect is not large enough to be of any practical significance.

Isocholesterol shows a very marked effect on the absorption of the dye. An examination of the raw data will show that its effect is nearly as great as that of cholesterol, and in one case (5% concentration in petrolatum) there is almost no significant difference between results from the two fractions. This phenomenon is difficult to explain, since there is no similarity between cholesterol and isocholesterol, the former being a sterol and the latter a mixture of two terpene alcohols, lanosterol and agnosterol (7, 8).

By far the largest increase in absorption is brought about by cholesterol and wool fat. The results show no significant difference at the 10% concentration; at the 5% concentration the difference is significant only at 20% level of significance and may therefore be considered doubtful. This would appear to be true if we take into consideration the facts that cholesterol normally constitutes about 20% of the wool fat and that the remaining 80% also enhances absorption, though to a lesser degree than does cholesterol.

It will be noted that wool fat when used as a base by itself causes greater absorption than when it is incorporated in petrolatum in concentrations of 5% and 10% and in polyethylene glycol ointment in a concentration of 5%. However, when 10% of wool fat is added to polyethylene glycol ointment, the additive effect of wool fat and the base becomes apparent and the absorption is greater than that caused by wool fat alone.

Cholesteryl Esters.—Cholesteryl palmitate and cholesteryl acetate increase the absorption of the dye from the polyethylene glycol ointment in a 5%

TABLE II

Base	Substance Incorporated	Concn. of Incorporated Substance, %	Mg. PSP Recovered from 10 Rats		
			Min.	Max.	Av.
PET	...	..	0	0	0
P. E. G. O.	...	..	0.09	0.115	0.0975
W. F.	...	..	0.20	0.30	0.25
PET	W. F.	5	0.050	0.175	0.101
PET	W. F.	10	0.20	0.225	0.211
P. E. G. O.	W. F.	5	0.15	0.16	0.154
P. E. G. O.	W. F.	10	0.32	0.375	0.339
PET	Cholesterol	5	0.10	0.16	0.134
PET	Cholesterol	10	0.20	0.225	0.21
P. E. G. O.	Cholesterol	5	0.15	0.225	0.1875
P. E. G. O.	Cholesterol	10	0.32	0.385	0.35
PET	Isocholesterol	5	0.10	0.17	0.128
PET	Isocholesterol	10	0.18	0.22	0.19
P. E. G. O.	Isocholesterol	5	0.15	0.21	0.17
P. E. G. O.	Isocholesterol	10	0.27	0.33	0.30
PET	Aliphatic alcohols	5	0.075	0.1	0.09
PET	Aliphatic alcohols	10	0.105	0.145	0.125
P. E. G. O.	Aliphatic alcohols	5	0.14	0.165	0.154
P. E. G. O.	Aliphatic alcohols	10	0.195	0.225	0.215
PET	Residue	5	0.04	0.06	0.05
PET	Residue	10	0.065	0.09	0.079
P. E. G. O.	Residue	5	0.12	0.18	0.14
P. E. G. O.	Residue	10	0.15	0.20	0.17
PET	Cholesteryl stearate	5	0	0.05	0.025
PET	Cholesteryl stearate	10	0.07	0.09	0.08
P. E. G. O.	Cholesteryl stearate	5	0.09	0.115	0.10
P. E. G. O.	Cholesteryl stearate	10	0.14	0.165	0.155
PET	Cholesteryl palmitate	5	0.04	0.07	0.053
PET	Cholesteryl palmitate	10	0.08	0.17	0.122
P. E. G. O.	Cholesteryl palmitate	5	0.13	0.21	0.17
P. E. G. O.	Cholesteryl palmitate	10	0.20	0.25	0.233
PET	Cholesteryl acetate	5	0.025	0.04	0.03
PET	Cholesteryl acetate	10	0.045	0.06	0.05
P. E. G. O.	Cholesteryl acetate	5	0.13	0.17	0.15
P. E. G. O.	Cholesteryl acetate	10	0.19	0.23	0.21

concentration; however, the effect of cholesteryl acetate does not become evident at that concentration but appears at the 10% concentration.

Cholesteryl palmitate has the greatest effect; cholesteryl stearate and cholesteryl acetate have the second largest and third largest effect, respectively. The effect of cholesteryl palmitate is exceptionally high at 5% concentration in the ointment of polyethylene glycol. At that concentration there is no significant difference between the value obtained from it and from cholesterol. However, at the 10% concentration in the same base, the mean value for cholesterol is much higher than that for cholesteryl palmitate. This would indicate that above the 5% concentration the effect of cholesterol on absorption increases more sharply than does the effect of the ester.

Effect of Concentration.—There is a highly significant difference between the values at the 5% and 10% concentrations. The values are usually double at the 10% concentration but this does not follow in all cases. It is likely that further expansion of the investigation employing different concentrations, such as 2.5%, 7.5%, 12.5%, etc., would better indicate the effect of concentration. It is quite possible that the pattern may vary with the nature of the incorporated substance.

Effect of Different Bases.—This study shows that absorption from polyethylene glycol ointment is definitely greater than from petrolatum, confirming the findings of previous workers. In fact, in this investigation petrolatum as such showed no absorption at all.

At the 5% concentration in polyethylene glycol ointment, quite often the results do not show a significant difference. This tendency is not present at the 10% concentration, indicating that at the lower concentration in this base, the effects of many of the substances tested are not fully defined.

TABLE III

In Polyethylene Glycol Ointment	In Petrolatum
Cholesterol and wool fat	Cholesterol and wool fat
Isocholesterol	Isocholesterol
Cholesteryl palmitate	Aliphatic alcohols
Aliphatic alcohols	Cholesteryl palmitate
Cholesteryl acetate	Residue
Residue	Cholesteryl stearate
Cholesteryl stearate	Cholesteryl acetate

The substances tested in each base have been arranged in decreasing order of effectiveness in causing absorption; see Table III.

CONCLUSIONS

Wool fat and its fractions, as well as the esters tested, enhanced the absorption of phenolsulphonphthalein from petrolatum and from polyethylene

glycol ointment; however, the increase in absorption caused by the cholesteryl esters, aliphatic alcohols, and residue was not large enough to be of any practical importance.

When combined with polyethylene glycol ointment, the substances tested should be used in a concentration of 10%, since at the lower concentration they do not fully manifest their individual effects.

The use of cholesterol does not offer any advantage over the use of wool fat; there was no significant difference in their results.

Polyethylene glycol ointment definitely is a better agent than petrolatum for causing the absorption of phenolsulfonphthalein.

No absorption could be detected from petrolatum in a twenty-four-hour period under the experimental conditions of this study.

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The Acetylation of Ephedrine in Dilute Aqueous Solution*

By LLEWELLYN H. WELSH

The acetylation of ephedrine in about a one per cent aqueous solution by means of acetic anhydride in the presence of sodium bicarbonate has been studied. Yields of 96 to 97 per cent of N-acetylephedrine may be obtained by using approximately 3.6 moles of anhydride per mole of ephedrine, but for the reaction to proceed in a literally quantitative manner, four times that amount of anhydride is required, and the reagent must be added portionwise. Yields are adversely affected by sodium acetate and by a large excess of bicarbonate. The influence of these agents is discussed.

PREVIOUS WORK in this laboratory showed that N-acetylephedrine may be obtained in 98 to 99 per cent yield by the action of acetic anhydride on a dilute (ca. 1%) aqueous solution of ephedrine in the presence of sodium bicarbonate (1). The procedure was applied to the analytical separation of ephedrine from procaine, and it was suggested that acetylation under such conditions might prove generally useful in the quantitative separation of ephedrine and related drugs from tertiary amines with which they might be compounded.

A shortage of 1 to 2 per cent below a theoretical recovery figure is of little practical importance if, as in the previous work, one is concerned only with the isolation of the ephedrine derivative from the reaction mixture. However, that shortage might be of significance if it represents unacetylated ephedrine and if one is interested also in quantitatively obtaining the pure tertiary amino component of the mixture. Thus, if a product were to contain ephedrine and a tertiary amine in a 5:1 ratio, 2 per cent of unacetylated (basic) ephedrine would eventually manifest itself as 10 per cent of impurity in the basic fraction containing the tertiary amine.

It was desirable, therefore, in order to improve the efficiency of the acetylation process, to study modifications of the somewhat arbitrarily selected conditions previously employed (1). Information obtained from such a study also might permit a better understanding of the fundamental factors involved in acetylating substances in dilute aqueous solution. Therefore the following theoretical considerations should be taken into account.

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In a system containing water, acetic anhydride, and an acylable organic substance, *e. g.*, an amine, two reactions occur simultaneously: acetylation of the substance (Eq. 1) and hydrolysis of the anhydride (Eq. 2).

$$RNHR^1 + Ac_2O \xrightarrow{v_A} RNAcR^1 + AcOH \quad (1)$$

$$HOH + Ac_2O \xrightarrow{v_H} 2AcOH \quad (2)$$

The velocities of the acetylation and hydrolysis reactions at any instant may be symbolized by v_A and v_H, respectively. According to principles of mass action, each of these should have a maximum value initially when the concentration of reactants is highest, and each should continuously decrease as the reactions proceed and the concentration of reactants becomes progressively smaller. Factors which increase the ratio v_A/v_H should increase the yield of acetylation product obtainable from a given quantity of anhydride. In order that a quantitative yield of product be obtained, the quantity of anhydride used must obviously be in excess of that stoichiometrically equivalent to the amount of acylable substance.

It is desirable to have present in the system a base which will neutralize the acetic acid formed, and thus allow the amine to retain its most easily acylable (free) form, shown in Eq. 1. Sodium bicarbonate is particularly useful for this purpose in pharmaceutical analysis, since by maintaining the *p*H of the system near the neutral point, it does not effectively promote, as does strong alkali, the decomposition of sensitive substances[1] by atmospheric oxygen.

EXPERIMENTAL

Reagents.—Sodium bicarbonate and acetic anhydride (A. C. S., reagent grades) were employed. The anhydride was Merck & Company material ("suitable for microanalysis") the purity of which was established as being 95% at the time of this work.

The strength of the anhydride was found to be important, particularly in those reactions in which the smaller quantities of anhydride were employed. Thus, under the conditions of experiment 4, Table I, anhydride having an assay value of 85% afforded an average yield of 92.8% instead of the average yield of 96.8% obtained by the use of a 95% reagent.

Procedure.—Samples of ephedrine hydrochloride (150.0 mg., 0.744 millimole) were dissolved in 10.0 cc. (556 millimoles) of distilled water contained in a 125-cc. separatory funnel. Sodium bicarbonate was added and the funnel was swirled until the bicarbonate had dissolved, or, in the experiments involving larger amounts of the reagent, until the solution had become saturated. Acetic anhydride was rapidly injected into the funnel by means of a calibrated 1-cc. hypodermic syringe; the funnel was immediately stoppered and vigorously shaken until the evolution of carbon dioxide had ceased (four to five minutes; shaking was interrupted momentarily several times to release pressure in the system). After the reaction mixture had been allowed to stand five minutes, it was acidified with 10% (w/v) sulfuric acid (10 cc., experiments 1–14; 15 cc., experiment 15; 20 cc., experiments 16, 17) and extracted with six 20- to 25-cc. portions of chloroform. Each extract was shaken in a second funnel with a solution of 0.6 Gm. of bicarbonate in 6 cc. of water, and then was filtered through a pledget of cotton. Removal of solvent, heating of residues, etc., was carried out as previously described (1).

Residues of acetylephedrine obtained melted at 84.5 to 85.5° (corr.) after being induced to crystallize. The pure recrystallized substance melts at 85.5 to 86.5° (3).

RESULTS

In the experiments which were conducted, the quantities of water and ephedrine were kept constant while the amounts of anhydride and bicarbonate were varied as indicated in Table I.

In the presence of an amount of bicarbonate about 10% in excess of that required to neutralize the acetic acid formed (experiments 1–4), yields of acetylephedrine did not approach a virtually quantitative figure (96.8% av.) until the quantity of anhydride was approximately three and six-tenths times the equivalent of the ephedrine present (experiment 4). Doubling this quantity of acylating agent (experiment 14) increased the average yield about 0.5%, while quadrupling it (experiment 16) raised the yield an additional 1%. A literally quantitative yield (99.9% av.) was obtained under the conditions of experiment 17 which differed from those of experiment 16 only in that the anhydride was added in three approximately equal portions instead of being added in one lot.

The results of experiments 4–14 show that, within wide limits, excess of bicarbonate does not exercise an appreciably adverse effect on yields. Thus in experiments 7–9, the yield is constant within experimental reproducibility when the excess is within the range of 10–35%. Even when the excess is raised to 56%, an increment of 21%, the yield is not materially affected (experiment 10). However, two additional 21%-increments of excess (experiments 11, 12) are associated with a decrease in yield of ca. 3% per increment.

DISCUSSION

From the experimental results, it is evident that as the quantity of acetylating combination (anhydride and a proportionate amount of bicarbonate) is increased, the percentage of anhydride utilized by the acetylation reaction is decreased. In experiment 1, for example, 53% of the 0.10 cc. of anhydride is

[1] By application of the technique described herein, quantitative yields of acetylation products having a high degree of purity have been obtained from the following substances: *o*- and *p*-aminophenol, catechol, pyrogallol, hydroquinone, morphine, epinephrine, and phenylephrine. In each case, phenolic hydroxyl groups and primary and secondary amino groups were acylated, whereas the secondary alcoholic functions of the latter three compounds were unaffected.

On a preparative scale, where the concentration of reagents is high and reaction is almost instantaneous, sodium hydroxide has been used with excellent results by Chattaway (2) in acetylating such substances.

TABLE I.—ACETYLATION OF EPHEDRINE (0.744 MILLIMOLE) WITH ACETIC ANHYDRIDE IN THE PRESENCE OF WATER (556 MILLIMOLES) AND SODIUM BICARBONATE

Experiment No.	Ac_2O, Cc.[a]	$NaHCO_3$, Mg.	$NaHCO_3$ Excess, %[b]	Yield Acetyl Ephedrine, %	Ac_2O Acylating, %
1	0.10	190	10	76.0	53.3
2	0.15	288	10	90.5	42.3
3	0.20	386	10	94.0, 93.5	32.9
4	0.25	484	10	96.7, 97.0 97.4, 96.1	27.2
5	0.25	563	31	96.6	27.1
6	0.25	1000	145	83.9	23.6
7	0.30	582	10	96.9	22.7
8	0.30	663	27	97.3	22.8
9	0.30	700	35	96.8	22.6
10	0.30	800	56	95.5	22.4
11	0.30	900	77	92.4	21.6
12	0.30	1000	99	89.1	20.8
13	0.40	1000	44	95.8	16.8
14	0.50	1000	13	98.2, 97.3 97.0, 97.3	13.7
15[c]	0.50	1000	13	88.6, 86.0	12.3
16	1.00	1950	10	98.2, 98.8 98.2, 98.6	6.9
17	1.00[d]	1950	10	99.7, 100.6 98.8, 100.4	7.0

[a] Added in one portion unless otherwise noted: 1 cc. of pure Ac_2O = 10.6 millimoles.
[b] In the calculation of these figures, correction has been made for the amount of bicarbonate required to liberate ephedrine base from the 150-mg. samples of hydrochloride employed.
[c] System initially 1 *N* with respect to anhydrous sodium acetate.
[d] Ac_2O added in portions of 0.40, 0.30, and 0.30 cc.

utilized in acetylating ephedrine, whereas in experiment 2, 42% of the 0.15 cc. is utilized. From these figures it may be calculated that only 20% of the increment of anhydride is involved in acetylation. The data indicate that under the experimental conditions the ratio of the velocity of acetylation, v_A, to the velocity of hydrolysis, v_H, is not a relatively constant one, but changes in such a way that v_A/v_H diminishes in value progressively from an initial maximum as the reactions proceed in the system and the concentration of reactants (with the exception of water) become progressively lower. Such a phenomenon may be related in part to a difference in the kinetic orders of the two reactions.[2]

Evidence that it is at least partly related to a salt effect of sodium acetate, the concentration of which builds up as the reactions proceed, is provided by the results of experiments 14 and 15 which show that the yield, and therefore the average v_A/v_H, is lowered by the presence of the salt.[3]

Excess bicarbonate may diminish the average value of v_A/v_H by virtue of exercising a salt effect similar to that shown by sodium acetate. However, altering the ratio may be related at least partly to the fact that a large excess of bicarbonate significantly affects the *p*H of the system. This possibility is suggested by the observation that, under the conditions of experiment 7 (10% excess bicarbonate), the *p*H of the system ranged from 8.17 before the addition of anhydride to 6.34 after completion of the reaction, whereas under the conditions of experiment 12 in which the quantity of bicarbonate was about twice the calculated amount, the *p*H range was 8.11 to 7.75.[4]

The fact that the anhydride is less efficiently utilized in experiment 16 than in experiment 17 may be related to the following circumstance. In the former reaction, the volume of anhydride added at one time is relatively large; the reagent therefore may not dissolve practically instantaneously in the saturated bicarbonate solution, but may instead form a second (anhydride-water) liquid phase, having an appreciable life, in which the value of v_A/v_H is relatively small compared with that of the average ratio prevailing in the principal aqueous phase.

SUMMARY

A study has been made to determine conditions necessary for the quantitative conversion of ephedrine, in dilute aqueous solution, to its N-monoacetyl derivative.

Factors affecting acetylation in an aqueous medium have been discussed.

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[2] Change of the value of the expression v_A/v_H in the observed direction could result from a situation in which the acetylation reaction is a second order process, with a velocity dependent on the concentration of both ephedrine and anhydride, and the hydrolysis reaction is a first order process, the velocity of which (because of the large excess of water) is dependent only on the concentration of anhydride. Hydrolysis of the anhydride, however, probably occurs simultaneously by two mechanisms, one involving water molecules, the other involving hydroxyl ions.

[3] One way in which this ratio may be diminished is by increasing v_H. In this connection, it is pertinent to note that Szabo (4) has observed that the velocity of hydrolysis of acetic anhydride is increased by acetate ion. In this laboratory, evidence has been obtained that the powerfully adverse effect of sulfite (i.e., alfite) ion on the acetylation of ephedrine in aqueous bicarbonate solution (1) is largely, if not entirely, due to the ability of the ion to increase catalytically the hydrolysis rate of acetic anhydride.

[4] The possibility that the effect of sodium acetate likewise may be due in part to an ability of the salt to change the *p*H of the system appears to be ruled out by the observation that in experiments 14 and 15, the *p*H ranges were 8.09 to 6.85 and 8.12 to 6.92, respectively. The measured difference in ranges is not regarded as being experimentally significant.

Bioassay of Cathartics. I. Emodin Type*

By ALBERT R. LATVEN,† AARON B. SLOANE,† and JAMES C. MUNCH‡,§

Tests of cathartics on the usual types of laboratory animals were reviewed. The unsatisfactory results obtained with the emodin type of drugs led to the development of a satisfactory method of bioassay on rats. The DD_{50} was determined by administration to a significant number of animals at several dosage levels; the consistency of the expressed fecal matter was determined eighteen hours later. Suitable standards were developed for comparable bioassays of several cathartics of the emodin type. The precision of these studies was about 15 per cent.

THE EMODIN or anthraquinone cathartics include senna, cascara, aloes, rhubarb, and frangula; the first three of these are extensively marketed and are therefore of great commercial importance. Since the whole drug or extracts thereof are used, various attempts have been made to assay such preparations for potency in order to maintain uniformly effective products. Chemical, gravimetric, and chromatographic methods have been applied for such purposes but have proved to be inadequate, and the need for biological assay has been evident (1–12). A number of such bioassay methods have been published (13–15), each procedure re-emphasizing the difficulties previously encountered. A satisfactory bioassay procedure for senna has been recently developed (16–21), using the mouse as the experimental animal; a similar mouse procedure for the bioassay of cascara has also been published (19), although a number of other methods (22, 23) have since appeared. However, no simple satisfactory method exists for the bioassay of aloes or podophyllin. These investigations were undertaken, therefore, to study the action of these agents with the hope of developing a satisfactory bioassay procedure which would also be applicable to other cathartics of the emodin group, as well as to all cathartics in general.

EXPERIMENTAL

To ensure experimental significance, a single sample of aloes known to be effective in humans was used throughout the initial investigation. Suspensions of the extract were prepared in a colloidal solution containing 1% tragacanth and 2% dispersing agent (Vanderbilt Darvan #2) and were used within an hour following preparation in all instances; in the few experiments in which solutions were required, they were prepared by dissolving the extract in 67% ethanol, shaking thoroughly, filtering, and then adjusting the *p*H to 8.5 with NaOH to prevent precipitation on dilution.

Isolated Intestine Strips.—Segments of the duodenum and the ileum from both guinea pigs and mice were suspended in modified Locke-Ringer's solution by the usual method, and solutions of aloe of various concentrations were introduced. The only response observed was one of relaxation, and in no instance did tonus or contractions increase. Subsequently, these tissues showed a marked resistance to acetylcholine and to histamine, while little change in their sensitivity to barium chloride occurred. In view of the fact that these results did not conform with the usual therapeutic action of the agent, further experiments with the isolated gut were postponed.

Alimentary Passage Time.—Initial *in vivo* studies were concerned with the influence of aloe upon the normal rate of food passage through the gastrointestinal tract. A solution (5.0%) of carmine was administered by oral intubation to both mice and rats, at doses of 0.20 cc. and 0.50 cc., respectively; the interval between its administration and appearance in evacuated stools established the alimentary passage time. The average CPT (Carmine passage time) of a group of 34 normal mice was found to be 112 minutes; simultaneous administration of aloe with the carmine did not alter the CPT significantly, the average of a group of ten mice being 116 minutes after 300 mg./Kg. and 111 minutes after 1000 mg./Kg. In rats, the normal CPT was found to be 408 minutes and the simultaneous administration of 500 mg./Kg. of aloe did not alter this value significantly in a group of 10 animals.

Since it had previously been observed that the intraperitoneal or oral administration of urethan at doses of 200–2000 mg./Kg. inhibited the CPT of mice to greater than 400 minutes, investigations were undertaken to determine the influence of aloe upon this inhibition. The simultaneous administration of 1000 mg./Kg. of aloe was found to be entirely ineffective upon such urethan inhibition within a period of seven hours.

Similarly, the inhibition of fecal elimination caused by phenobarbital, 125 mg./Kg. orally, or by ANTU, 30 mg./Kg. orally, was unaffected within seven hours by 1000 mg./Kg. of aloe.

Time of Action.—To determine the time of laxative action of aloe in rats, a group of these animals was administered doses ranging from 10 to 400 mg./Kg. orally. At two-hour intervals following dosage, fecal samples were manually expelled or extruded for examination. It is a general observation that the rectum of the rat invariably contains one or more fecal pellets, making it possible to obtain samples by manual expulsion at almost any time, providing the animals are eating normally.

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To accomplish this, the rat is grasped with the left hand with the fingers across the back and the thumb pressing the base of the abdomen; with the forefinger and thumb of the right hand, the skin over the rectum is compressed as far forward as possible, and the contained fecal mass can then be forced out. The simplicity of this procedure permits the rapid removal of specimens from a large number of animals. Normal samples were found to be relatively hard, dry, and of a blackish color. No deviations from this appearance among aloe-injected animals occurred within seven hours following intubation. On the eighth hour, however, specimens were found to be of a brown to tan color; on the tenth hour, the samples were found to be soft also, and at the higher dosage levels, diarrhea was manifested by the presence of semi-liquid stools. The intensity of these effects became maximal at ten to twelve hours, decreasing slowly and progressively at later periods. Normalcy returned within twenty-four hours at the lower dosage levels, but continued for forty-eight or seventy-two hours at the higher dosage levels.

These observations were then extended to permit evaluation as a means of establishing a bioassay method. Although the ideal period of time elapsing after intubation for fecal examination was ten to twelve hours, it was recognized that this interval was quite undesirable as a routine procedure; based on the fact that only slight changes occurred between this optimal time and eighteen hours, the latter interval was, therefore, chosen as the most feasible and practical time for fecal examination.

Observational Criteria.—In order to determine a valid criterion for a laxative response, a large group of rats was injected orally with various dose levels of aloe. Fecal specimens were taken after eighteen hours and were critically examined by means of arbitrarily chosen criteria for the most uniform dose-response relationship.

It was found that the change in fecal color from black to brown to tan was the most sensitive criterion, this alteration becoming manifest at dosage levels well below those causing any change in stool consistency. Unfortunately, however, it was subsequently found that a large number of normal, untreated rats yielded brown fecal pellets on various days. Therefore, this criterion was discarded. (It is possible that another type of diet may overcome this variation.)

Fecal specific gravity, as a possible criterion, was investigated by placing the fecal pellets in beakers of distilled water and of salt solutions; the normal specimen floats in distilled water with the ease of a ping-pong ball, whereas a soft (laxative) specimen invariably sinks to the bottom, due to the absence of fermentation pockets and to the greater water content. Again, as with the color changes, this criterion was too sensitive an index, and excessive individual variations found among normal animals necessitated its discard because of unreliability.

Changes in the consistency of fecal specimens occurred after aloe dosage, ranging from soft to liquid, with various gradients between these extremes. Attempts were made to classify the response in terms of "soft or greater," "semi-liquid," and "liquid." On these bases, a definite dose-response relationship was found, and it was possible to calculate the median effective dose for each degree of response; the approximate ratio among the median doses required to produce soft, semi-liquid, and liquid stools was found to be approximately 1:2:6. However, it was frequently difficult, if not impossible, to ascertain the definite classification of borderline specimens because of the inherent subjectiveness of such observation.

A simple means of denoting changes in fecal consistency, which reduced the subjective factor to a minimum and increased accuracy without undue loss of experimental time, was found in a principle referred to herein as "wire adhesion." It was applied as follows: (*a*) the fecal sample, manually expelled from the rat, was permitted to fall upon a clean, oil-free glass plate; (*b*) within five minutes thereafter, a stiff wire was inserted vertically downward into the specimen; and (*c*) the wire was then lifted. Specimens of normal consistency invariably adhered to the wire and were lifted from the glass plate; however, "soft" stools adhered to the glass instead, probably by virtue of greater quantity of surface mucus as well as a greater contact area of adhesion. The method, therefore, resulted in "all or none" data, a most desirable criterion characteristic for bioassay purposes, by eliminating the various degrees of intensities of cathartic effect and thereby reducing observational subjectivity to a minimum.

Before adopting this method, wires of various diameters were evaluated for efficiency. These ranged from 0.34 to 0.90 mm. diameter; the latter (obtained from a 17G hypo needle) was found to yield the most consistent results and was used throughout subsequent studies.

BIOASSAY METHOD

Application of the foregoing observations has permitted the development of a bioassay method which, although designed for investigations with emodin cathartics, is applicable to the investigation of all agents producing this pharmocodynamic effect. The procedure involves the determination of the median defecatory dose (DD_{50}) for obvious purposes of accuracy and the data reported herein have been obtained as follows:

Male rats of a single strain (Charles River), kept under constant dietary conditions of Purina Fox Chow and water *ad libitum*, were used. To facilitate administration of the drugs, suspensions were prepared by initially triturating a weighed quantity of the substance with the inert dispersing agent Darvan #2[1] (to prevent "gumming-up") and the mixture was then triturated into homogeneous suspension by the gradual addition of a 1% solution of tragacanth; the final suspension always contained 1% tragacanth, 2% dispersing agent, and a variable concentration of the cathartic agent. Variously spaced dosage levels encompassing the entire dose-response curve were used, with four rats at each level. After examining individual fecal samples for normality, each test rat was weighed to the nearest gram and doses were calculated on the milligram per kilogram basis. Oral administrations were made through the use of a metal catheter passed directly into the stomach. After a specific interval of time (eighteen hours in the case of aloe), fecal samples

[1] Kindly supplied by R. T. Vanderbilt Co., Inc., New York, N. Y.

were manually expelled from the rectum and allowed to fall on a clean glass plate; a fine wire with a diameter of 0.90 mm. was inserted vertically downward into the fecal sample and then lifted; the sample was considered to be negative if it adhered to the wire and was considered to be positive if it adhered to the glass. Twenty to thirty minutes later a second fecal sample was taken and similarly examined; the maximum effect demonstrated by either of the samples was taken as the characteristic response. Behrens' method was then applied to the data to calculate the median defecatory dose.

The Median Defecatory Dose of Aloe.—With aloe, fecal specimens were examined eighteen hours after administration. Four separate determinations for the DD_{50} of aloe were made in male CR (Charles River) rats weighing 150–200 Gm. and the following values were found: 39, 38, 35, and 41 mg./Kg., with a combined average value of 38 mg./Kg. These data are shown in Table I and the results are plotted in Fig. 1. The repeatability of the assay using such relatively small numbers of animals was surprising in view of the shallow slope of the response curve. In order to ascertain whether marked differences in response occurred in another strain of animals, similar determinations were made with male Blaine-Wistar rats weighing 100–150 Gm.; the DD_{50} was found to be 55 and 52 mg./Kg. in two such tests, with a combined average value of 53 mg./Kg., thus

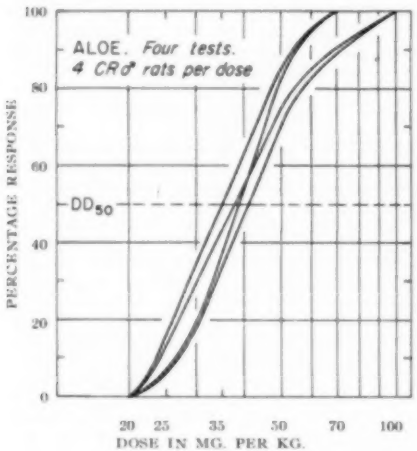

Figure 1.

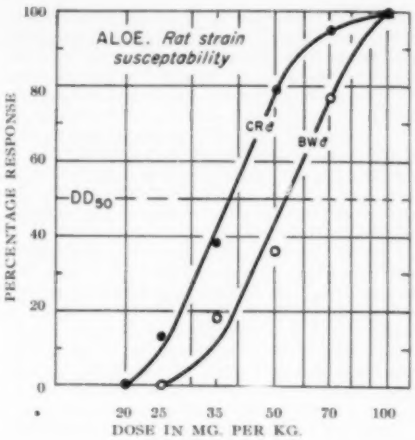

Figure 2.

indicating definite strain variation. These data, shown in Table II and Fig. 2, indicate a definite variation in response due to animal strain, although the response within a single strain appears to be reasonably uniform.

Since differences in susceptibility between male and female animals exist with many drugs, it was of interest to investigate this factor in regard to aloe. Using female BW rats (100–150 Gm.), the DD_{50} was found to be 92, 91, and 100 mg./Kg. in three successive tests, with a combined average of 94 mg./Kg. These data are presented in Table II and plotted in Fig. 3. Since the comparable value in males was 53 mg./Kg., it is apparent that female rats of this strain were almost twice as resistant to the action of aloe as the males. (A similar difference in sex susceptibility has been found with podophyllin, which will be reported at a later date.)

The Median Defecatory Dose of F. E. Cascara.—In order to determine the applicability of the method to cascara, initial investigations with this cathartic were concerned with the time required for the development of maximum physiological action. A group of rats was orally injected with suspensions of a fluidextract (prepared in accordance with U. S. P. XIII) at various dosage levels. Fecal samples taken at two-hour intervals and examined by the "wire-adhesion" method, revealed that the maximal effect occurred approximately twelve hours after administration, being similar in

TABLE I.—THE DD_{50} OF ALOE ADMINISTERED ORALLY TO MALE CR RATS

Dose, Mg./Kg.	1st Test			2nd Test			3rd Test			4th Test			5th Test		
	No. Neg.	No. Pos.	% Response*	No. Neg.	No. Pos.	% Response*	No. Neg.	No. Pos.	% Response*	No. Neg.	No. Pos.	% Response*	No. Neg.	No. Pos.	% Response*
20	4	0	0	4	0	0	4	0	0	4	0	0	16	0	0
25	3	1	13	3	1	13	3	1	14	3	1	11	12	4	13
35	3	1	33	2	2	43	2	2	50	3	1	29	10	6	38
50	1	3	83	1	3	75	1	3	86	1	3	71	4	12	79
70	0	4	100	1	3	90	0	4	100	1	3	89	2	14	95
100	0	4	100	0	4	100	0	4	100	0	4	100	0	16	100
DD_{50}	39 mg./Kg.			38 mg./Kg.			35 mg./Kg.			41 mg./Kg.			38 mg./Kg.		

* Calculated by Behrens' method.

this respect to the action of aloe. Subsequent to this interval, the intensity of the effect decreased with time. However, because of the long duration of action, it was possible to conduct fecal examinations at the more practical interval of eighteen hours following administration. Under these conditions the median defecatory dose of cascara was found to be approximately 0.26 cc./Kg. of the extract (representing 1 Gm. of whole drug per cc.).

TABLE II.—THE DD_{50} OF ALOE ADMINISTERED ORALLY TO BW RATS

Dose, Mg./Kg.	Male Rats, Combined Data No. Neg.	No. Pos.	% Response[a]	Female Rats, Combined Data No. Neg.	No. Pos.	% Response[a]
25	8	0	0	4	0	0
35	5	3	18	12	0	0
50	6	2	36	11	1	3
70	3	5	77	8	4	23
100	0	8	100	6	6	55
140	0	8	100	3	9	87
200	0	4	100	0	12	100
DD_{50}	53 mg./Kg.			94 mg./Kg.		

[a] Calculated by Behrens' method.

The Median Defecatory Dose of Senna.—Studies with senna revealed that the cathartic activity of this powder became maximal seven to nine hours (average 8.3 hours) after oral administration to male CR rats weighing 200–300 Gm. The duration of effect was much shorter than that observed with either aloe or cascara; after eighteen hours, the intensity of the effect as well as its incidence had decreased considerably, and within twenty-four hours only those animals receiving doses greater than the DD_{100} yielded soft stools. As a consequence, the interval of eight hours was chosen as the most practical observation time, all other factors being kept constant. Under these conditions the DD_{50} of senna in male CR rats weighing 200–300 Gm. was found to be 60 mg./Kg.

DISCUSSION

It is apparent on the basis of these studies that the most important factor in the bioassay of any cathartic is the time required for the production of a maximal effect. Because of this, a specific latent interval exists before any laxative effect is produced; this effect then increases in intensity to maximal, followed by a progressive decrease with time. As with most drugs, those substances which require a longer period of time to produce an effect also possess an action which is of longer duration; this permits a greater latitude in the time at which experimental observations can be made. Thus, the cathartic action of senna was found to be maximal in rats eight hours after administration, and was virtually dissipated within twenty-four hours. On the other hand, aloe and cascara produced their most intense cathartic effects in rats between ten and fourteen hours, an interval most inconvenient for observation under customary laboratory working conditions. However, the prolonged action of these substances permitted observations to be made at a later and more practical time; an eighteen-hour interval was found to be entirely satisfactory for the routine bioassay of these cathartics. It should be pointed out, however, that studies concerning comparative evaluations of different cathartics must be conducted at the particular action-time of each cathartic, if proper and precise quantitation is to be obtained.

When applied as a routine procedure, the assay method for the estimation of cathartic potency as described herein has been modified to conform with statistical design by the application of the two- and three-dose methods. The evaluation of a series of commercial powdered extracts of aloe by this procedure has revealed that, although many of these extracts were of "average" potency, occasional preparations were found to be half as effective and others twice as effective. These results emphasize the importance of assay in the maintenance of a uniformly potent product.

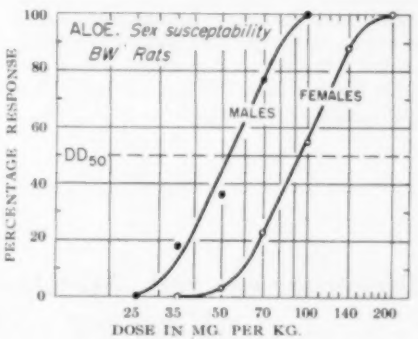

Figure 3.

In any bioassay, the evaluation of potency implies and requires a comparison with the potency of a known preparation. Unfortunately, the absence of any such "reference standard" for aloe and for other cathartics makes it necessary to adopt specific preparations to serve this purpose. To be of practical value the proper potency of the preparation so chosen should, of course, be representative of that particular substance, i. e., should represent the average potency of all such preparations, being neither more effective nor less effective than the average. This can best be accomplished by pooling a large number of different batches of the product for this service, or by adopting a single batch whose potency is the same as the average potency of a large number of batches. In any event, it is hoped that the active principle in pure form will be isolated from each of the emodin cathartics to serve this important purpose.

CONCLUSIONS

(1) A bioassay procedure for the evaluation of emodin compounds in rats has been developed and described.

(2) The period of time required to produce a maximal cathartic effect varies among the cathartics.

(3) Aloe produces a maximal cathartic effect in rats approximately twelve hours after administration. The median defecatory dose (DD_{50}) of aloe in male CR rats was found to be 38 mg./Kg. Male rats were found to be almost twice as sensitive to action of aloe as female rats. The sensitivity of rats to aloe varies among different strains of this species. Variations in the potency of different batches of aloe have been found ranging from 60 to 180 per cent of average aloe potency.

(4) Cascara produces a maximal cathartic effect in rats approximately twelve hours after administration. The median defecatory dose of F. E. cascara in male CR rats was found to be approximately 0.26 cc./Kg.

(5) Senna produces a maximal cathartic effect in rats approximately eight hours after administration. The median defecatory dose of powdered senna in male CR rats was found to be approximately 60 mg./Kg.

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Biosynthesis and Metabolism of Some Radioactive *Veratrum viride* Alkaloids*,†

By A. PIRCIO and E. M. K. GEILING

THE increased interest in the *veratrum* alkaloids is evident from the large number of papers which have recently been published on this subject. However, none of these investigations have dealt with the distribution throughout tissues and fluids of the body nor with the excretion pattern of these alkaloids. Such studies have not been carried out because a sensitive method for the quantitative detection of the *veratrum* alkaloids and their metabolites in body fluids, tissues, and excreta at clinical levels has been lacking.

This laboratory has been engaged in a radioactive biosynthesis program during the past six years during which time radioactive digitoxin (1), morphine (2), colchicine (3), and nicotine (4) have been prepared. Thus the method of biological synthesis has allowed the introduction of carbon-14 into the molecules of complex, medicinally important drugs. The introduction of a radioactive isotope has produced a sensitive method for the detection not only of the unchanged drug but also of its metabolic products.

The first part of the present investigation describes the biosynthesis of some of the *veratrum* alkaloids. The second part presents data on the excretion of two of the *veratrum* alkaloids, one hypotensively active (germidine) and the other hypotensively inactive (veratrosine).

EXPERIMENTAL

Methods.—*Veratrum viride* roots which had been wintered were allowed to develop about 6–9 inches of leaf growth. The plants were then individually bottled as previously described (1). Carbon dioxide was injected into the enclosed atmosphere surrounding the plant in 100-cc. portions every other day. The carbon dioxide contained 0.1 mc. of carbon-14 per 100 cc. of gas so that each plant received a total of 1.5 mc. in a period of one month. At the end of this growing period the radioactive plants were harvested, dried at room temperature

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† This work was done under a contract between the Atomic Energy Commission and The University of Chicago, and in part under a grant from the Medical Life Insurance Research Fund.

in an open atmosphere for four weeks, and finally ground into powder by means of a Wiley Mill.

Jervine, veratramine, and veratrosine were prepared by the methods of Jacobs and Craig (5) while the newer procedure of Fried, *et al.* (6) was employed for the isolation of germidine.

These alkaloids have the following physical properties:

Jervine: m. p. 240–241°; $[\alpha]_D^{28} = -147°$ (in absolute ethanol); specific activity = 18,250 C. P. M./mg.

Veratramine: m. p. 208.5 to 210°; $[\alpha]_D^{28} = -68°$ (in methanol); specific activity = 19,125 C. P. M./mg.

Germidine: m. p., sintered at 235° and melted between 243–245°; $[\alpha]_D^{28} = -55°$ (in equal volumes of ethanol and chloroform); specific activity = 27,700 C. P. M./mg.

An excretion study was undertaken with radioactive germidine and veratrosine. The germidine solution was made by dissolving the alkaloid in several cubic centimeters of 5% acetic acid and diluting to the proper volume with normal saline solution and acetic acid so that the final solution contained 1.0% acetic acid. The veratrosine solution was prepared by dissolving the glycoside in an excess of glacial acetic acid and titrating to a *p*H of 5.5 with $NaHCO_3$. Final concentration of these two alkaloidal solutions was 500 γ per cc. of solution.

Ten adult male rats of the Sprague-Dawley strain, weighing between 150–225 Gm., were used in this experiment. Five animals were injected intraperitoneally with 500 γ of radioactive germidine (11,525 counts/min.) and five with 500 γ of radioactive veratrosine (13,850 counts/min.). The rats were then placed in metabolism cages for collection of urine and feces. The excreta were collected every twenty-four hours for five days and analyzed for two radioactive fractions.

The feces were homogenized in 6 volumes of water, carefully alkalinized with 20% sodium hydroxide at 5° to *p*H 8.0 and then with sodium carbonate to *p*H 10.0. The alkaline solution was added into a separatory funnel and shaken 3 times with twice the volume of chloroform. The combined chloroform extract was concentrated *in vacuo* to approximately 20 cc. An aliquot of the chloroform solution was pipetted directly on weighed oxidized copper disks for measurement of radioactivity. This sample represents the chloroform-soluble fraction of the feces. The alkaline layer was neutralized and centrifuged. The residue was washed twice with warm distilled water and the wash added to the supernatant liquid. An aliquot of the supernatant liquid was treated as above. This sample represents the water-soluble fraction of the feces.

The urine samples were treated exactly as described for the treatment of the stools except that the urine was not diluted before alkalinization.

A preliminary study of the metabolic products in the chloroform fraction of both the feces and the urine was made by means of descending paper chromatography. Aliquots of the pooled and concentrated urine chloroform fractions and of the pooled and concentrated stool chloroform fractions were placed in $^1/_2$-in. transverse bands on strips of Whatman No. 1 filter paper. The chromatograms were developed in the following system: ether:acetic acid:water (50:5:25). When the solvent front had moved 13–14 in. past the initial band, the chromatograms were air dried and cut transversely into $^1/_4$-in. sections, each of which was eluted with chloroform, plated on oxidized copper disks, and measured for radioactivity.

All aliquots were plated and corrected for self-absorption by the method of Calvin, *et al.* (7). Radioactivity measurements were made with a windowless gas-flow type of Geiger-Müller counter as described by Kelsey (8). Specific activities were determined by plating a known amount of the substance to be assayed on oxidized copper disks and measuring its radioactivity.

RESULTS AND DISCUSSION

The daily excretion of compounds containing carbon-14 after the intraperitoneal injection of radioactive germidine and veratrosine is presented in Figs. 1 and 2, respectively. The most striking fact in the excretion data is the high activity found in the feces as compared to the urine. In the case

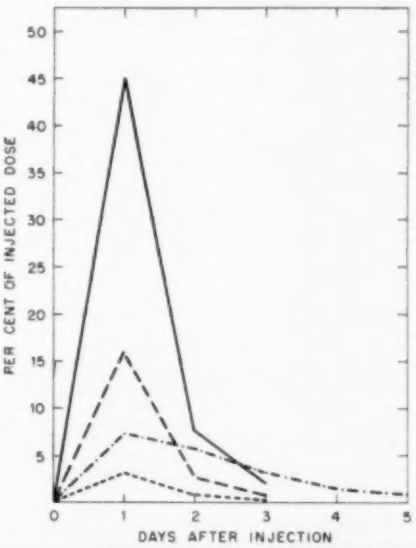

Fig. 1.—The daily fecal and renal excretion of C-14 after injection of 500 γ of radioactive germidine. The data represent an average of data obtained on five rats. ——— Feces (water-soluble fraction); — — — Feces (chloroform-soluble fraction); —·—· Urine (water-soluble fraction); - - - - Urine (chloroform-soluble fraction).

of both germidine and veratrosine, over 40% of the activity was excreted by way of the feces during the first twenty-four hours after administration. The greater part of the activity found in the feces was in the form of water-soluble metabolites. These data suggest that the *veratrum* alkaloids are principally metabolized in the liver and eliminated by the bile. Of course, another contributing source to the high fecal activity is possible; i. e., excretion through the mucosa of the alimentary canal.

Further investigation will be needed to clarify this point on gastro-intestinal excretion.

The excretory data also show that the compounds containing carbon-14 are rapidly excreted by the rat, since more than 85% of the injected radioactivity in both cases appears in the excreta twenty-four hours after administration. By the fifth day, only about 1% of the activity injected can be found; this activity resides in the water-soluble fraction of the urine. Thus the carbon-14, injected as radioactive germidine and veratrosine, is completely eliminated by five days after injection.

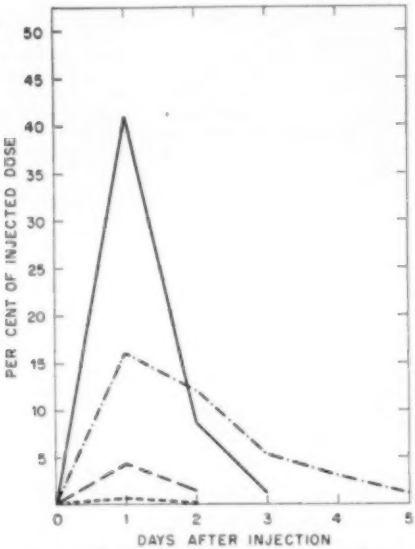

Fig. 2.—The daily fecal and renal excretion of C-14 after injection of 500 γ of radioactive veratrosine. The data represent an average of data obtained on five rats. ——— Feces (water-soluble fraction); — — — Feces (chloroform-soluble fraction); —·—·— Urine (water-soluble fraction); – – – – Urine (chloroform-soluble fraction).

A preliminary study of the metabolic products in the chloroform fractions of the pooled urine and feces was attempted. The chloroform fraction was chosen because any unchanged alkaloid present in the feces or urine would be found in this fraction. The location of the radioactivity on the strip was determined by means of a Geiger-Müller counter as described above. The R_F values of the various radioactive components located in this way are tabulated in Table I.

The chromatograms indicate the presence of at least four radioactive components in the excreta in the case of germidine and five radioactive components in the excreta of veratrosine-treated animals. In both the germidine and veratrosine experiments a radioactive band with an R_F identical with that of the pure alkaloid was found only in the chloroform fraction of the feces, thus indicating the presence of unchanged alkaloid. However, the amount of unchanged alkaloid represents less than 3% of the injected alkaloid.

TABLE I.—R_F VALUES OF VARIOUS RADIOACTIVE COMPONENTS IN CHLOROFORM FRACTIONS OF POOLED EXCRETA

	Germidine		Veratrosine	
	No. of Band	R_F	No. of Band	R_F
Chloroform fraction of pooled radioactive urine	1	0	1	0.560
	2	0.703	2	0.902
Chloroform fraction of pooled radioactive feces	1	0.520	1	0
	2	0.632	2	0.320
			3	0.940
Chloroform fraction of pooled normal feces plus radioactive germidine	1	0.626	..	...
Chloroform fraction of pooled normal feces plus radioactive veratrosine	..	...	1	0

SUMMARY

1. The biosynthesis technique has been employed for the preparation of radioactive *Veratrum viride* alkaloids.

2. Excretion and metabolic studies have been carried out on rats with two radioactive *Veratrum viride* alkaloids, germidine and veratrosine.

3. The data suggest that these two alkaloids are metabolized rapidly in the animal, particularly by the liver, and excreted chiefly as water-soluble metabolites, appearing mostly in the feces.

4. The presence of four radioactive components in the excreta in the case of germidine and five in the cast of veratrosine was indicated by paper chromatography.

5. Less than three per cent of the injected alkaloids were excreted unchanged.

6. The excretion pattern of germidine, a hypotensively active *Veratrum viride* alkaloid, was quite similar to that of veratrosine, a hypotensively inactive *Veratrum viride* alkaloid.

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The Toxicity and Distribution of 'Ilotycin'*,†

By ROBERT C. ANDERSON, PAUL N. HARRIS, and K. K. CHEN‡

'Ilotycin' is a crystalline antibiotic produced by an actinomycete, identified as a strain of *Streptomyces erythreus*. It has shown significant activity against pathogenic Gram-positive organisms and against some of the more important Gram-negative organisms such as the *Neisseria, Hemophilus,* and *Brucella* groups. Acute toxicity tests by different routes of administration to each of several species of animals have shown the antibiotic to be of low toxicity, which has been confirmed by subacute studies now underway. 'Ilotycin' has been found in the blood, urine, feces, and cerebrospinal fluid of dogs which had received the drug by mouth. The antibiotic is basic; numerous salts have been prepared, the acute toxicity of several of which are reported. Blood and urine levels following parenteral injections to dogs are included.

'ILOTYCIN' is a crystalline antibiotic produced by an actinomycete, *Streptomyces erythreus* (Waksman). McGuire, *et al.* (1) have shown in a preliminary report that 'Ilotycin' is active against pathogenic Gram-positive and some of the more important Gram-negative organisms such as *Neisseria, Hemophilus,* and *Brucella* groups. 'Ilotycin' has also shown activity against viruses of the lymphogranuloma and mouse meningopneumonitis type, rickettsiae, and some animal parasites. This study is concerned with acute, subacute, and chronic toxicity studies and with the absorption and excretion in experimental animals. The pharmacodynamics, methods of assay, studies of antibacterial, antiviral, antirickettsial, and antiparasitic activity, and clinical results will be reported elsewhere.

In various experiments, 'Ilotycin' was used as either the base or the hydrochloride. The base is soluble in water only to the extent of 0.2 per cent at room temperature. A homogeneous suspension containing 100 mg./ml. was prepared by triturating 'Ilotycin' carefully with water. This was suitable for injection into experimental animals. The hydrochloride is soluble to the extent of 4 per cent. 'Ilotycin' base used in these studies assayed 900 μg./mg.; the hydrochloride assayed 830 μg./mg.

EXPERIMENTAL

Acute Toxicity.—'Ilotycin', as both the base and the hydrochloride, was administered by different routes to mice, rats, hamsters, guinea pigs, rabbits, and dogs, with the object of determining the median lethal dose (LD_{50}). Results obtained with the base are shown in Table I. When lethal and sublethal doses were given by mouth, mice either became prostrate and died within twenty-four hours, or completely recovered. No pathological changes could be detected in the parenchymatous organs of the dead animals. Because of the excessive volume necessary, the median lethal doses could not be determined in rats, either orally or subcutaneously. Like mice and rats, hamsters can also tolerate large amounts of 'Ilotycin', the LD_{50} exceeding 3 Gm./Kg. It is obvious from the results with rodents that 'Ilotycin' as a base has a low toxicity.

The results obtained with the hydrochloride of 'Ilotycin' are given in Table II. The hydrochloride, given orally, also has a low toxicity toward mice and rats. By intravenous injection, 'Ilotycin' hydrochloride is approximately twice as toxic to rats and rabbits as to mice. The intraperitoneal LD_{50} is only slightly larger than the intravenous LD_{50}. Guinea pigs also are susceptible to intraperitoneal injection of the base (Table I).

Intravenous injection of lethal and sublethal doses in all species caused clonic convulsions, prostration, and respiratory depression. Dogs developed violent tremors during the intravenous injection, which prevented the completion of the LD_{50} determination. Defecation was frequently observed during the injection. All animals either died during the first hour or recovered completely. The surviving rabbits and dogs were sacrificed after one week and submitted for necropsy. No visceral damage could be found in any animal.

Subacute Toxicity in Rats.—Female rats in 3 groups of 5 each, weighing 70 to 90 Gm., were fed diets containing 0.05, 0.10, and 0.20% 'Ilotycin' for three months, following the procedure reported previously from this laboratory (2). Comparison of the growth curves of each group with that of a control group are shown in Fig. 1. The average daily food intake per rat was practically constant for each group: 14.43, 13.43, 14.79, and 13.35 Gm. for the 0.05, 0.10, 0.20%, and control groups, respectively. It is apparent (Fig. 1) that 'Ilotycin' did not alter the growth curve of rats fed concentrations as great as 0.2% in the diet for three months.

One rat fed 0.05% in the diet died after sixty-four days from acute peritonitis and pleuritis. Another rat was killed accidentally while a blood sample was being taken on the seventy-first day of treatment. One rat in the control group died after

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† Eli Lilly and Co. has registered 'Ilotycin' (Erythromycin, Lilly) as the trademark of this antibiotic.

‡ We wish to thank D. W. Ziegler and C. E. Higgins for the various assays performed; Miss Nila Maze for the hematologic studies; and Mrs. Marian Small, Charles L. Rose, Robert D. Fink, Harold Worth, and John Owen for their assistance in the many experiments reported.

TABLE I.—ACUTE TOXICITY OF 'ILOTYCIN' (AS THE BASE)

Species	Mode of Administration	$LD_{50} \pm S.E.$, Mg./Kg.
Mouse	Oral	3112 ± 211
	Subcutaneous	>2500
Rat	Oral	>3000
	Subcutaneous	>2000
Guinea Pig	Intraperitoneal	413.4 ± 51.7
Hamster	Oral	3018 ± 190

thirty days with acute peritonitis and slight hydrothorax. All others survived the three-month period, and were sacrificed and subjected to necropsy. Gross and microscopic studies of the heart, lungs, liver, spleen, pancreas, stomach, intestines, kidneys, thymus, thyroid, and adrenal glands revealed no abnormalities.

Hemoglobin values were determined, and erythrocyte, leucocyte, and differential counts were made weekly in the usual manner. No differences were apparent between the treated and the control groups. Some hemo-concentration and the usual fluctuation in leucocyte counts were seen. There was no indication at any time of leucocytic destruction.

Subacute and Chronic Toxicity in Dogs.—Twenty mongrel female dogs were selected from a large series after control hematopoietic studies and urine analyses had shown them to be essentially normal. They were divided at random into 4 groups of 5 dogs each, and were given daily doses of 50, 75, and 100 mg./Kg., respectively, the fourth group serving as a control. The daily dose was divided so that half of it was administered soon after 8 a. m. and the remainder shortly before 5 p. m. All doses were based on the initial weight and were dispensed in capsules.

All treated dogs survived the three-month test period. Two control dogs died, one after twenty days from pneumonia and the other after forty days with a perforated duodenal ulcer and peritonitis. After thirteen weeks 3 dogs from each treated group and 2 controls were sacrificed and submitted for necropsy. The daily dose was increased to 100 mg./Kg. for the surviving 6 dogs. Studies on these and on one control will be continued for an indefinite period. Table III lists each dog number, the daily dose, the effect on body weight, and the status at the time of this report.

The 11 dogs sacrificed were submitted for complete necropsy. A control dog, No. 472, had some fibrosis of the medulla of the kidney. Some tubules in the cortex and medulla contained hyaline eosinophilic casts. Dog No. 433, which received 50 mg./Kg. daily, had minute granulomata in the lungs, liver, and adrenals; two of the granulomata in the adrenals had necrotic centers suggestive of tuberculosis. Dog No. 504 on the same dosage regime had a scar on the left kidney. Dog No. 499, on 75 mg./Kg. daily, had large fat globules in many liver cells and a minute granulomatous nodule in the adrenal cortex. Otherwise, the gross and microscopic examination of the heart, lungs, liver, spleen, stomach, pancreas, intestines, kidneys, and adrenal glands were normal in all dogs. Apparently none of the previously mentioned abnormalities had any relationship to the antibiotic.

In addition, bone marrow punctures were made in each dog at three sites (the rib, tibia, and iliac crest) at the time of sacrifice. No abnormalities were found in any marrow. The counts of myeloid, erythroid, and lymphoid cells were comparable to those of the control dogs.

TABLE II.—ACUTE TOXICITY, THE HYDROCHLORIDE OF 'ILOTYCIN'

Species	Mode of Administration	$LD_{50} \pm S.E.$, Mg./Kg.
Mouse	Intravenous	425.6 ± 15.7
	Intraperitoneal	490.0 ± 30.4
	Subcutaneous	1849 ± 89
	Oral	2927 ± 162
Rat	Intravenous	209.4 ± 12.6
	Subcutaneous	1442 ± 144
	Oral	>2000
Rabbit	Intravenous	Est. 183
Dog	Intravenous	>140

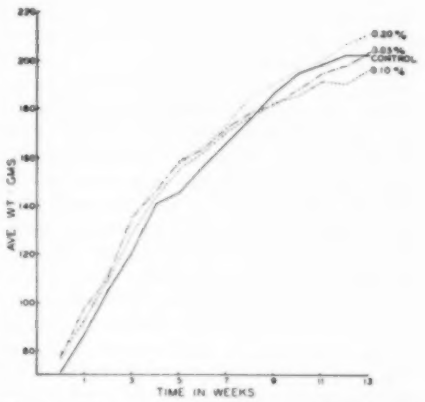

Fig. 1.—Effect on the growth curve of female rats that received 'Ilotycin' in the diet for three months.

Blood and urine samples were taken weekly from each dog for various analyses. Hemoglobin levels, erythrocyte, leucocyte, and differential counts showed normal variations. There was no suggestion of leucocytic destruction at any time. Whole blood clotting time had a mean of 19.6 minutes at the start of the experiment and a mean of 20.5 minutes at the end of thirteen weeks. Control nonprotein nitrogen values ranged from 25.2 to 54.7 mg./100 cc. of blood. After thirteen weeks, the range was 26.6 to 45.9 mg./100 cc. Blood sugar determinations showed no alteration; the control levels varied from 57 to 104, and after thirteen weeks the values were 56 to 84 mg./100 cc. of blood. In this laboratory, a fluctuation of ±20 mg. of glucose per 100 cc. of blood is not considered significant. A trace of sugar was found in the urine of 10 dogs prior to treatment; in 11 dogs it appeared after thirteen weeks of treatment. The majority of the dogs had traces of albumin in the urine at the start of and during the study. This is probably due to the high protein diet consumed. One of

TABLE III.—DOGS ORALLY DAILY

Dog Number	Daily Dose, Mg./Kg.	No. of Doses	Body Weight, Kg. Initial	Body Weight, Kg. Terminal	Status
209	50	91	7.6	12.2	On test
433	50	91	5.6	7.1	Sacrificed
456	50	91	10.0	11.3	Sacrificed
504	50	91	6.3	7.0	Sacrificed
509	50	91	8.2	8.1	On test
448	75	91	7.8	9.4	Sacrificed
489	75	91	5.8	7.2	Sacrificed
498	75	91	8.5	10.5	On test
499	75	91	6.5	10.0	Sacrificed
535	75	91	4.2	5.1	On test
481	100	91	8.5	9.2	On test
484	100	91	5.0	5.8	On test
486	100	91	7.4	9.4	Sacrificed
490	100	91	8.0	10.4	Sacrificed
528	100	91	5.8	6.1	Sacrificed
472	Control	..	5.9	5.9	Sacrificed
479	Control	..	8.4	9.1	Sacrificed
488	Control	..	5.4	3.5	Died
495	Control	..	7.6	9.6	On test
497	Control	..	9.4	8.0	Died

the control dogs had some hematuria which was caused by mechanical damage to the urethra on catheterization.

Neurotoxic Studies with Cats.—Following Molitor's procedure (3), 'Ilotycin' was studied for possible damaging effect to the eighth cranial nerve. Six cats were injected intramuscularly with daily doses of 50 mg./Kg., using a 2.5% solution of the hydrochloride. The hind legs were alternated as the sites of injection. Four other cats were treated at the same time with streptomycin, given as the sulfate. Practically all animals lost weight during the first twenty-four days on a diet of milk and canned dog food. When the latter was replaced by salmon or mackerel, the weight losses ceased, and in two cats a weight gain was evident. The results are summarized in Table IV. All of the streptomycin cats vomited within thirty minutes after dosage for the first few days of treatment, but this effect subsided. Only 2 cats of the 'Ilotycin' group vomited, each only once, approximately five weeks after the start of the experiment.

Periodically, all animals were tossed to a height of approximately one meter as recommended by Molitor. All those that received 'Ilotycin' were able to right themselves at all times before landing. There was no nystagmus or wobbling gait, although the large daily volume (4–5 cc.) of the antibiotic caused some stiffness in the hind leg muscles. Two of the cats on streptomycin were ataxic and staggered and fell when taken from their cages. They were unable to right themselves in midair when tossed as previously described. Both were sacrificed after 27 daily doses.

All cats were submitted for necropsy at death or on sacrifice. Cat No. 1, which received 27 doses of streptomycin, had necrosis of a few ganglion cells in the medulla oblongata. The other cat, No. 4, which was sacrificed after 27 doses of streptomycin, had necrosis of a few ganglion cells in the pons and medulla. The sections of the internal ears appeared normal in both. The cause of death was not evident in cats No. 2, 3, and 8. Cat No. 5 had some pulmonary edema. Cat No. 7 died over the week end, and post-mortem changes obscured the results. Cats No. 6, 9, and 10 which were sacrificed after 69 doses of 'Ilotycin' had fibrosis of the muscles at the sites of injection. Otherwise there was no apparent visceral damage. In contrast to streptomycin, 'Ilotycin' may be said to have no deleterious effect on the eighth cranial nerve in cats.

TABLE IV.—CATS INTRAMUSCULARLY DAILY

Drug	Cat Number	Change of Body Weight, Kg.	No. of Doses	Results
Streptomycin	1	−1.027	27	Sacrificed
	2	−0.489	9	Died
	3	−0.216	4	Died
	4	−0.534	27	Sacrificed
'Ilotycin'	5	−0.313	5	Died
	6	−0.487	69	Sacrificed
	7	−1.044	66	Died
	8	−0.269	9	Died
	9	+0.403	69	Sacrificed
	10	+0.253	69	Sacrificed

Absorption and Excretion in Dogs.—To determine the blood concentrations obtained with single doses of 'Ilotycin,' 4 dogs were given oral doses of 50 mg./Kg. Blood and urine samples were collected hourly, and cerebrospinal fluid was collected at intervals of one, three, and five hours after dosage. Similar studies were made with the same doses of terramycin and aureomycin. Serum and cerebrospinal fluid levels of 'Ilotycin' and terramycin were determined by a modification of the Rammelkamp assay method for penicillin, using a hemolytic streptococcus (C203) as the test organism (4). A broth dilution test with *Bacillus cereus* (Lilly—X143) as the test organism was employed

for aureomycin serum and cerebrospinal fluid assays. Urine samples were analyzed for 'Ilotycin' or terramycin by a turbidimetric method employing *Micrococcus pyogenes* var. *aureus*, and for aureomycin by a similar method using *Klebsiella pneumoniae* as the test organism. The serum levels are compared in Fig. 2 and the urinary excretion in Fig. 3.

As can be observed from Fig. 2, the peak of the serum level of 'Ilotycin' was the highest of the three and arrived earlier than that of the other two antibiotics. The levels of all three were still measurable after eight hours. Figure 3 shows that the urinary excretion of 'Ilotycin' was highest during the first two hours; then it fell below that of terramycin. The excretion of aureomycin was the lowest at all hours. The excretion results coincide well with the serum levels.

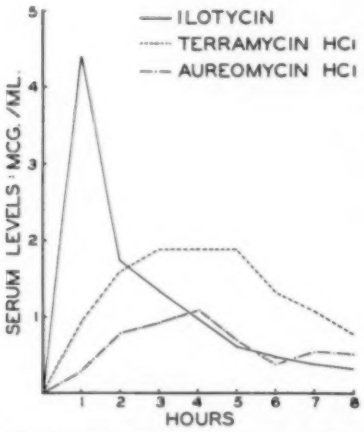

Fig. 2.—Average serum levels in dogs after oral doses of 50 mg./Kg. of 'Ilotycin,' aureomycin, and terramycin.

'Ilotycin' traverses the blood-brain barrier of dogs. The cerebrospinal fluid taken one hour after dosage contained 0.04, 0.16, 0.16, and 0.64 μg./ml. respectively. Three- and five-hour samples were slightly lower, with 3 of the 4 dogs having measurable levels after five hours. Dogs that received aureomycin also showed detectable quantities in the spinal fluid. One-hour samples had <0.008, <0.008, 0.008, and 0.038 μg./ml. respectively with levels of 0.019 to 0.038 μg./ml. present after five hours. All 4 dogs given terramycin failed to show detectable quantities within the five-hour test period (<0.3 μg./ml.).

The results obtained with terramycin and aureomycin in dogs are comparable to those in patients as reported by Welch (5) and Herrell (6). Welch found higher blood and urine levels with terramycin than with aureomycin when larger doses were used. Herrell concluded that "unlike aureomycin, terramycin did not readily traverse the blood-brain barrier." Our results appear to confirm their observations.

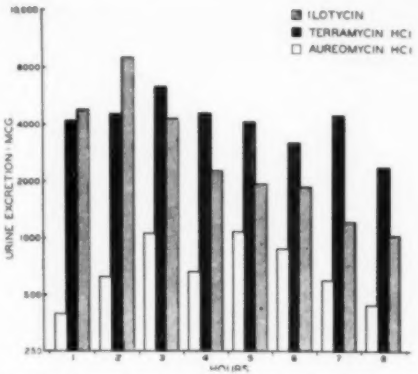

Fig. 3.—Average urinary excretion in dogs following oral doses of 50 mg./Kg. of 'Ilotycin,' aureomycin, and terramycin.

SUMMARY

1. The acute toxicity of 'Ilotycin' has been determined by different routes of administration in various laboratory animals (mice, rats, hamsters, guinea pigs, rabbits, and dogs). 'Ilotycin' has a low toxicity by mouth. Intravenously, it is much more toxic than by subcutaneous or oral administration. The intraperitoneal LD_{50} is only slightly larger than the intravenous LD_{50}. Death was preceded by clonic convulsions, prostration, and respiratory depression.

2. Subacute toxicity studies have been carried out in rats and dogs. Rats fed diets containing concentrations of 0.05 to 0.2 per cent for three months had no pathological changes in the viscera. Similarly, dogs tolerated daily doses of 50 to 100 mg./Kg. for more than three months. The formed elements of the blood in both rats and dogs were not altered. In dogs, the marrow constituents were normal, as were the blood and urine analyses.

3. Cats receiving 'Ilotycin' intramuscularly in the daily dose of 50 mg./Kg. showed no evidence of eighth nerve damage after sixty-nine days. Two cats, receiving the same dose of streptomycin, developed signs of vestibular involvement within four weeks.

4. 'Ilotycin,' like terramycin and aureomycin, is absorbed when given by mouth. The peak of the blood concentration is more quickly reached than with terramycin or aureomycin. It appears to be as rapidly excreted as terramycin. Like aureomycin, 'Ilotycin' gains entrance to the cerebrospinal fluid in dogs.

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The Effect of Four Antiheparin Dyes on the Isolated Ileum and Uterus*,†

By JAMES L. LEITCH and THOMAS J. HALEY

BECAUSE of the small amount of information available on the pharmacodynamic effects of toluidine blue, azure A, neutral red, and neutral violet and on the use of toluidine blue in the treatment of the hemorrhagic syndrome produced as a consequence of whole body irradiation or nitrogen mustard therapy (1–4), the pharmacological properties of these dyes on isolated organs have been investigated. The quantitative results obtained with the guinea pig ileum indicate that the dyes have a very low antispasmodic potency compared to atropine. It was not possible to quantitate the results obtained with the rabbit ileum, and the rabbit uterus work indicated that toluidine blue and azure A stimulated this organ while neutral red and neutral violet depressed it.

EXPERIMENTAL

The dyes, toluidine blue (2-methyl-3-amino-6-dimethylaminophenothiazine chloride), azure A (3-amino-6-dimethylaminophenothiazine chloride), neutral red (2-methyl-3-amino-6-dimethylaminophenazine HCl) and neutral violet (2-dimethylanilinoamino-3-amino-6-dimethylaminophenazine HCl) were from the same lots used previously (5, 8). The dosage ranges employed varied from 0.01 γ/cc. to 70 γ/cc., depending upon the particular dye and its solubility. The atropine dosage range was from 0.001 γ/cc. to 200 γ/cc. The doses of spasmogens used on the ileum preparations are given in Tables I and II. The isolated tissues were studied by the Magnus method in a bath maintained at 37.5° ± 0.2°. Tyrode's solution was prepared as directed by Jackson (6) and had a *p*H of 8.1 to 8.2. Addition of drugs did not change this *p*H value. All drugs were added to the bath in a constant volume of 0.5 cc. To obtain rabbit uteri at the same stage of estrus, New Zealand rabbits were given one daily injection of 1 γ of α-estradiol for three days followed by a similar treatment with 1 γ of progesterone for the next three days. The rabbits were sacrificed two days after the last injection, and the responses of the uteri were studied by the Magnus method. Where possible, the data obtained were analyzed statistically by the Litchfield-Wilcoxon method (7).

RESULTS

Guinea Pig Ileum.—Table I gives the calculated ED_{50} values of the dyes and of atropine. These data indicate that all of the dyes have an extremely low activity against acetylcholine spasm compared to atropine and only toluidine blue is comparable to the latter in counteracting pilocarpine spasm. However, all of the dyes except neutral violet are equivalent to atropine in blocking nicotine spasm.

Rabbit Ileum.—With the spontaneously contracting rabbit ileum, it was possible to study the effect of the four dyes on the rate and amplitude of contraction. Little or no change in the rate of contraction was observed with any of the dyes until complete paralysis occurred. The amplitude was unaffected by any of the dyes at a concentration of 0.1 γ/cc., but above that concentration and up to and including a concentration of 60 γ/cc., neutral red and neutral violet produced a continuously decreasing amplitude approaching complete paralysis. Furthermore, repeated washing with fresh Tyrode's solution did not restore normal contractility. In contrast, toluidine blue and azure A increased the amplitude approximately 50% at a concentration of 10 γ/cc. and then slowly decreased it, until at a concentration of 50 γ/cc. the amplitude was only slightly higher than initially. The spasmogens did not influence these direct effects of the dyes, and the results obtained are given in

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TABLE I.—ANTISPASMODIC ACTIVITY OF FOUR ANTIHEPARIN DYES ON ISOLATED GUINEA PIG ILEUM

Drug	Acetylcholine, 2.5×10^{-2} γ/cc. ED_{50} and Range,[a] γ/cc.	Pilocarpine, 1 γ/cc. ED_{50} and Range,[a] γ/cc.	Nicotine, 1 γ/cc. ED_{50} and Range,[a] γ/cc.
Atropine sulfate	4.25×10^{-4} (2.15×10^{-4}–8.39×10^{-4})	0.22 (0.08–0.59)	1.7 (0.40–7.2)
Toluidine blue	8.6 (4.91–15.1)	0.71 (0.11–4.65)	8.7 (3.42–22.1)
Azure A	19.0 (8.71–41.5)	2.80 (0.93–8.40)	5.4 (1.05–27.7)
Neutral red	8.6 (5.49–13.5)	6.80 (2.84–16.2)	3.4 (1.04–11.1)
Neutral violet	9.0 (4.76–16.9)	5.10 (2.14–12.1)	10.5 (4.3–25.6)

[a] Range at 19/20 confidence limits.

TABLE II.—INHIBITION OF THE ACTION OF ACETYLCHOLINE, PILOCARPINE, AND NICOTINE BY FOUR ANTIHEPARIN DYES ON THE RABBIT ILEUM

Drug	Acetylcholine, 2.5×10^{-2} γ/cc. Drug Concn., γ/cc.	Inhibition, %	Pilocarpine, 2.5 γ/cc. Drug Concn., γ/cc.	Inhibition, %	Nicotine, 0.5 γ/cc. Drug Concn., γ/cc.	Inhibition, %
Atropine sulfate	1.0	0.0	0.100	0.0	100.0	0.0
	1.25	100.0	0.125	61.0	200.0	100.0
	...	...	0.25	100.0	...	...
Toluidine blue	10.0	0.0	25.0	0.0	50.0	0.0
	50.0	16.0	50.0	30.0	...	...
Azure A	10.0	0.0	50.0	0.0	50.0	0.0
	50.0	59.0	...	...	...	...
Neutral red	10.0	0.0	10.0	0.0	12.5	0.0
	50.0	100.0	60.0	100.0	25.0	50.0
	...	...	...	...	60.0	100.0
Neutral violet	10.0	0.0	25.0	0.0	25.0	0.0
	50.0	100.0	60.0	59.0	60.0	100.0

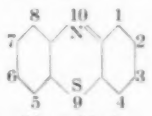
Table II. Irreversible staining of the tissue occurred because even prolonged washing with fresh Tyrode's solution would not remove the color from the tissues.

Rabbit Uterus.—As a test for normal contractility, each uterine segment was tested with a dose of 0.2 unit of α-hypophamine and only those responding with a vigorous contraction and change in tone were used. At doses of 0.01 and 0.1 γ/cc., the dyes had no effect upon the rate or amplitude of uterine contractions. At doses of 1.0 to 50 γ/cc. both toluidine blue and azure A increased the rate of contraction and gave a tonus change similar to that obtained with the α-hypophamine. At the same dosage range, neutral violet had no effect on the rate of contraction but decreased the amplitude 40%. No tonus changes were observed. Neutral red, at doses from 10 to 50 γ/cc., had a variable effect upon the uterus, either paralyzing the organ or not changing its rate and amplitude of contraction. The isolated uterus was irreversibly stained by all of the dyes, and continued washing did not remove the dye which had stained the cells. However, even when the uteri were stained they still reacted to α-hypophamine as vigorously as initially, showing that the dyes were incapable of blocking the direct musculotropic action of this hormone.

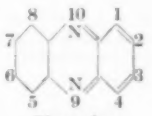
DISCUSSION

Leitch and Haley (8) reported that all four of the dyes had a slight atropine-like action on the frog heart but not on the mammalian heart. The present evaluation of antispasmodic activity, using the guinea pig ileum, would confirm this atropine-like action. However, the dyes are approximately 10,000 times less potent than atropine in blocking acetylcholine and therefore cannot be considered effective antispasmodic agents. On the other hand, the dyes are similar to atropine in that they have a more potent antimuscarinic (block pilocarpine) than antinicotinic effect.

There appears to be a correlation between chemical structure and pharmacological effect when the data obtained on the spontaneously contracting rabbit ileum and uterus are considered. The phenothiazine dyes (toluidine blue and azure A) are muscle stimulants, whereas the phenazine dyes (neutral red and neutral violet) are depressants. Although there are changes in the various radicals attached to the ring and in the size of the aliphatic groups on the amino nitrogen atoms, these variations do not appear to have as much influence on pharmacological activity as the changes in position 9 of the basic nucleus.



Phenothiazine



Phenazine

The sulfur atom apparently confers stimulant properties on the compound, whereas the nitrogen

atom confers depressant properties. This is particularly evident when a comparison is made between toluidine blue and neutral red.

SUMMARY

The antispasmodic properties of four antiheparin dyes have been investigated. It was found that these dyes were about 10,000 times less potent than atropine in counteracting acetylcholine spasm of the guinea pig ileum. They have a less potent antimuscarinic activity than atropine, but with the exception of Neutral Violet, are equivalent to atropine in their antinicotinic effects. There appears to be a definite relationship between their chemical structure and their effects on the rabbit ileum and uterus.

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An Antibacterial Substance from the Leaves of *Arbutus menziesii*[*,†]

By E. ROY HAMMARLUND[‡], DERROL E. PENNINGTON[§], and L. WAIT RISING[¶]

A procedure is presented for extracting and concentrating a relatively nontoxic and water-soluble antibacterial agent from the leaves of *Arbutus menziesii*. The chemical, physical, and biological characteristics of the substance were investigated with the main emphasis on its antibacterial properties against human pathogens. In this respect, the principle is mainly active *in vitro* against the Gram-positive and acid-fast bacteria and shows very little activity against most of the Gram-negative bacteria.

IT HAS BEEN discovered in this laboratory that the leaves of the Pacific Madrona tree (*Arbutus menziesii*, family *Ericaceae*) contain an antibacterial substance of considerable potency. The following is an account of the extraction and concentration procedures, and the physical, chemical, and biological properties of the substance. The name "madronin" is used to designate the active material.

EXPERIMENTAL METHODS

Assay.—The detection and evaluation of the antibacterial activity of extracts of the Madrona leaves were made by observing the effect of these extracts on the growth of cultures of *Micrococcus pyogenes* var. *albus*. Two methods of assay were used—one, a quantitative serial dilution method, and the other a modification of Vincent's (1) qualitative filter paper disk diffusion plate method for determining presence or absence of antibacterial activity.

In the serial dilution method, the fraction tested was dissolved in 10 ml. of distilled water and sterilized by means of a Swinny filter, only the last 5 ml. being collected. This sterile solution was diluted with 9 parts of sterile distilled water for assay.

To sterile tubes containing 5.0 ml. of the appropriate medium were added 1.0, 0.6, 0.4, 0.3, 0.2, 0.1, and 0.05 ml. of the test solution, and sterile water was added to each tube to make its final volume 6.0 ml. A tube containing 6.0 ml. of sterile water only was used as a blank. The series of assay tubes thus contained from 2.5 μg. to 50 μg. of sample per ml.

The inoculum which was added to each tube was prepared from a twenty-four hour nutrient broth culture of *M. pyogenes* var. *albus* diluted 1 to 10,000 with sterile distilled water. A drop (0.05 ml.) of this dilution, which contained approximately 5 x 10^4 viable cells, was added to each of the tubes.

The contents of each tube were mixed by swirling, and the tubes were incubated at 37° for twenty hours. The greatest dilution which gave a complete inhibition of growth of the inoculum was considered the end point of the titration. The amount of extract required was calculated as μg. per ml. for complete inhibition at twenty hours.

The medium used was adjusted to pH 6.8 to 7.0; it was a modification of Evans' (2) synthetic

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medium for *Staphylococcus* and had the following composition:

	Weight in Mg. per 100 Ml. Medium
Glucose	500
Casein hydrolysate	250
Yeast extract	50
K_2HPO_4	100
dl-Tryptophane	1
l-Cystine	1
$MgSO_4 \cdot 7H_2O$	2
NaCl	1
$FeSO_4 \cdot 7H_2O$	0.1
$MnSO_4 \cdot 4H_2O$	0.1

In the diffusion plate method, small sterile disks of filter paper were thoroughly moistened in the concentrated aqueous solution to be tested, and were then placed on nutrient agar plates which had been heavily seeded with 1 ml. of a twenty-four hour broth culture of the microorganism in question. The plates were then incubated at 37° for twenty hours. The presence of a definite zone of inhibition of growth of any size surrounding the paper disks indicated antibacterial activity. The size of the zone of inhibition may be interpreted as a semi-quantitative measure of the concentration of the extract.

Extraction Procedure.—The antibacterial substance was readily extracted from the leaves by water and by various alcohols. The following illustrates the procedure developed and used most frequently in this laboratory.

Fresh leaves (100 Gm.) from Madrona trees were cut into small pieces and repeatedly extracted in a Waring Blendor with successive 200-ml. portions of ethyl ether until the extract became nearly colorless. From three to five extractions were usually necessary, and the leaf residue was filtered off and saved for further extraction. The green ether extract contained very little antibacterial activity and was discarded.

The ether-insoluble leaf residue contained the major portion of the activity and was extracted twice in a blendor with 150-ml. portions of 85% ethanol. The mixture was centrifuged and the alcohol extract containing the active principle was decanted and evaporated to dryness under vacuum.

The dried alcoholic extract was then dissolved in 100 ml. of water and filtered, yielding a clear, golden-tan extract of the antibacterial principle.

Concentration Procedure.—The active aqueous extract was de-ionized by passing through cation (IR 100-H) and anion (IRA-400) exchange resin columns. The active substance is neutral and remained in the effluent which was then evaporated to dryness from a frozen state under reduced pressure. This procedure effected a two- to threefold concentration of the activity with essentially no loss.

The resulting dried extract (approx. 7.0 Gm.) was finely powdered, and then was repeatedly extracted in a large centrifuge tube with hot acetone. The mixture was centrifuged, and the butter-yellow acetone solution of the active principle was decanted and evaporated to dryness under reduced pressure, yielding approximately 4.5 Gm. of material. The white, acetone-insoluble residue (approx. 2.5 Gm.) was inactive and thus was discarded.

The dried acetone extract was dissolved in a minimum amount of absolute ethanol (approx. 10 ml.) and passed through a descending 1-in. by 10-in. chromatographic adsorption column packed with $CaHPO_4$. Three separate yellow zones were formed on the column by developing and eluting with absolute ethanol. The lowest yellow zone (which contained the largest portion of the active principle) was collected as it was washed from the column, filtered, evaporated to dryness under reduced pressure, and powdered. This powder (approx. 2.5 Gm.), which inhibited the test organism at a concentration of 4.5 μg./ml., was used in all of the characterization tests.

The two remaining yellow zones could also be successively eluted from the column by use of 75% ethanol and then 50% ethanol. However, their removal from the column did not prove practical since they contained only a relatively small amount of activity. The above procedure yielded a relatively concentrated but still non-crystalline product which represented about 2.5% of the original weight of fresh leaves.

Although the leaves were the most abundant source, the same procedure was used to extract activity on a qualitative basis from the inflorescence that is obtainable in the Northwest during the months of May and June. None could be extracted from the bark of the tree. Other plant parts were not investigated in this study.

No further increase in activity could be obtained by any of the following methods: partition chromatography, using water-saturated butanol as the solvent on a silica gel partition column moistened with HCl; adsorption chromatography, using activated alumina, talc, and magnesium trisilicate in place of $CaHPO_4$ as the adsorbent; charcoal adsorption, using activated charcoal (Darco G-60 and Norit A); ether precipitation, by pouring an acetone or alcohol solution of madronin into 5 volumes or more of ethyl ether; heavy metal precipitation, using saturated aqueous solutions of lead acetate and mercurous nitrate; and attempted crystallization from organic solvents, using methanol, ethanol, methylethyl ketone, methylpropyl ketone, tetrahydroxyfuran, 1,4-dioxane, ethyl lactate, formamide, glacial acetic acid, and pyridine.

EXPERIMENTAL RESULTS

Physical and Chemical Characteristics.—Madronin is a slightly hygroscopic, light yellow, noncrystalline powder. It has a slightly sweet, astringent taste. It is organic in nature and does not contain nitrogen, sulfur, halogen, phosphorus, or ash. It does not exhibit a sharp melting point, but slowly darkens above 200° and chars at approximately 250°.

Madronin is very soluble in water, methanol, and ethanol. It is fairly soluble in acetone, glacial acetic acid, formamide, 1,4-dioxane, tetrahydrofuran, butanol, methylethyl ketone, methylpropyl ketone, and ethyl lactate.

Madronin is practically insoluble in amyl alcohol, ethyl acetate, ethyl ether, chloroform, benzene, petroleum benzin, and camphor.

It appeared to be nearly neutral in reaction and did not form salts readily. It also demonstrated neutral characteristics when passed through resin ion-exchange columns and when undergoing electrophoresis.

Various qualitative chemical tests were conducted to determine the presence or absence of characteristic functional groups:

The carbohydrate is absent, as indicated by negative Molisch and fermentation tests.

Alkoxy groups are absent, as indicated by a negative Zeisel test.

Madronin exhibits strong reducing properties, shown by positive Benedict, Baeyer, and Tollen reduction tests.

Since 2,4-dinitrophenylhydrazones and *p*-nitrophenylhydrazones could not be obtained and the Fuchsin and Methone tests also were negative, the presence of an aldehyde group appears unlikely unless it is sufficiently hindered to produce negative characterization tests.

The ferric chloride, phthalic anhydride, nitrous acid, and Millon test for phenolic groups gave either negative or doubtful reactions.

Negative ceric nitrate and molybdic acid tests indicated that hydroxy groups are most likely absent.

The saponification equivalent was determined as 168. A neutralization equivalent was not clearly defined since there appeared to be a masked acidic group present, possibly in the form of a lactone.

Paper Partition Chromatography.—Madronin has been characterized by means of R_F values obtained with six binary mixtures of solvents, using a modification of the ascending paper partition chromatographic method of Williams and Kirby (3). The position of the active material on the dried paper strips was determined by punching out filter paper disks at 1-cm. intervals along the entire strip of paper from the spot where the sample was applied to the edge of the solvent front. These disks were then plated in the same order as punched on a *M. pyogenes* var. *albus* seeded agar plate, using the diffusion plate method previously described. There were sizable zones of inhibition of growth around those disks which were impregnated with the active material. The size and position of the active spots were then traced on the chromatogram strip, and the R_F value was calculated for the center of each active spot. The calculated R_F values of madronin active principles, using various solvent mixtures, are found in Table I. A control consisting of a blank strip of paper was developed in each solvent mixture in a similar manner and gave no inhibition when plated.

Ultraviolet Absorption.—The ultraviolet absorption of aqueous solutions of madronin was measured in a Beckman spectrophotometer. The solutions used for analysis contained 16 μg. of madronin per ml. Figure 1 was obtained by measuring the ultraviolet absorption from 350 mμ to 210 mμ at *p*H 4.0, 6.0, 8.0, and 10.0. Acetate, phosphate, and H_3BO_3-NaOH-KCl mixtures were used as the corresponding buffer solutions.

The madronin solution darkened in color as the *p*H was raised to 8.0 and 10.0. The absorption changes were reversible, and when the solution was readjusted to *p*H 6.0, the original absorption curve resulted.

TABLE I.—R_F VALUES OF MADRONIN PRINCIPLES IN VARIOUS SOLVENT MIXTURES

Solvent	R_F Values
Water saturated butanol	0.25;[a] 0.70
Water saturated amyl alcohol	0.10;[a] 0.52
Water-methylethyl ketone (50:50)	0.83
Water-methylpropyl ketone (50:50)	0.10;[a] 0.44
Water saturated ethyl ether	0.12
Water saturated benzene	0.00

[a] Indicates the major fraction.

The $E^{1\%}_{1\ cm.}$ in solution at *p*H 6.0 was approximately 250 at 275 mμ.

Specific Rotation.—Both a 1% aqueous solution of madronin and a 1% solution of 0.1 *N* HCl gave a specific rotation of approximately −23° at 25° in monochromatic sodium light.

Moisture Content.—When powdered madronin which had been previously vacuum-dried for an hour at room temperature (25°) was slowly heated in an oil bath at 150° under reduced pressure, a loss in weight of 7.5% resulted. Numerous bubbles formed from the dry powder at the bottom of the tube as the temperature was slowly raised over 110°. There was no carbonization or darkening of the sample at this temperature, and after being cooled, the dried material retained its antibacterial property.

Stability.—All of the stability determinations were conducted by using the serial dilution method, and the samples in question were compared to a fresh solution of madronin for a control.

Madronin was stable in acidic or neutral solutions and in weakly alkaline solutions. A phos-

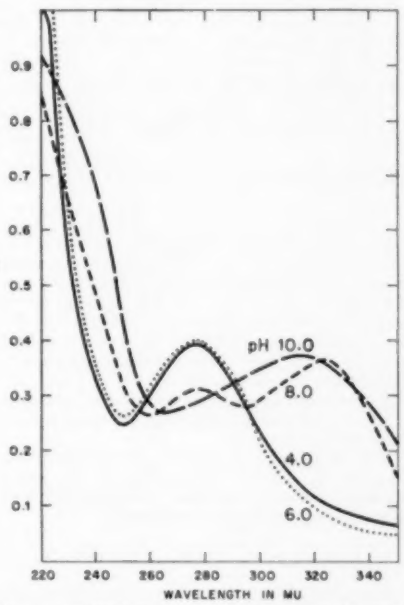

Fig. 1.—Ultraviolet absorption spectrum of madronin at various *p*H levels.

TABLE II.—ACTIVITY OF MADRONIN AGAINST VARIOUS MICROORGANISMS

Organism	Medium	Inoculum Dilution	Inhibiting Concn., μg./Ml.
Aerobacter aerogenes	Nutrient broth	10^6	No inhibition
Alkaligenes faecalis	Nutrient broth	10^6	8.0
Bacillus megatherium	Nutrient broth	10^6	4.0
Bacillus mycoides	Nutrient broth	10^4	12.0
Bacillus polymyxa (30°)	Nutrient broth	10^4	8.0
Bacillus subtilus	Nutrient broth	10^6	4.5
Brucella abortus (40 hr.)	3% Serum broth	10^4	No inhibition
Brucella melitensis (40 hr.)	3% Serum broth	10^4	No inhibition
Brucella suis (40 hr.)	3% Serum broth	10^4	No inhibition
Candida albicans	Sabouraud agar	Plate	No inhibition
Clostridium novyi	Thioglyco. broth	10^4	8.0
C. diphtheriae	Nutrient broth	10^6	8.0
C. hoffmani	Nutrient broth	10^4	8.0
D. pneumoniae (type III)	3% Serum broth plus glucose	10^2	No inhibition
D. pneumoniae (type III)	3% Serum broth plus agar	Plate	Good[a]
Escherichia coli	Nutrient agar	Plate	No inhibition
Gaffkya tetragena	Nutrient broth	10^6	3.0
Klebsiella pneumoniae (type A)	Nutrient broth	10^6	15.0
Kloeckera brevis	Sabouraud agar	Plate	No inhibition
Leuconostoc citrovorum	Yeast extract, glucose	10^4	No inhibition
Mycobacterium phlei (60 hr.)	2% Glycerol	10^4	11.0
M. smegmatis (60 hr.)	2% Glycerol	10^4	11.0
M. tuberculosis (6 days) (H37Rv)	3% Serum, Proskauer & Beck	10^2	No inhibition
M. tuberculosis (6 days) (H37Rv)	3% Serum agar	Plate	Good[a]
Micrococcus pyogenes var. albus	Staph. broth	10^6	4.5
M. pyogenes var. aureus	Staph. broth	10^6	11.0
Neisseria catarrhalis	Nutrient broth	10^4	60.0
Paracolon species	Nutrient agar	Plate	No inhibition
Proteus vulgaris	Nutrient broth	10^4	60.0
Pseudomonas aeruginosa	Nutrient agar	Plate	No inhibition
Saccharomyces cereviseae	Sabouraud agar	Plate	No inhibition
Salmonella paratyphi	Nutrient broth	10^4	No inhibition
Salmonella shottmuelleri	Nutrient broth	10^4	No inhibition
Salmonella typhosa (H&O)	Nutrient broth	10^4	No inhibition
Sarcina lutea (40 hr.)	Staph. broth	10^2	4.5
Serratia marcescens	Nutrient agar	Plate	No inhibition
Shigella dysenteriae	Nutrient broth	10^6	8.0
Shigella paradysenteriae	Nutrient broth	10^4	8.0
Streptococcus faecalis	Staph. broth	10^4	11.0
Streptococcus hemolyticus	3% Serum broth	10^4	20.0
Streptococcus viridins (SBE)	Nutrient agar, glucose	Plate	No inhibition

[a] Inhibition zone diameter of 15–25 mm.

phate buffered solution of madronin was boiled for five minutes at pH 8.0 with no loss in activity. Strong alkaline solutions, however, caused considerable loss of activity. A solution of madronin in 0.1 *N* NaOH, boiled for ten minutes and then neutralized, lost one-half of its activity. A solution in 0.1 *N* HCl, boiled for the same length of time, did not lose any of its activity.

Autoclaving an aqueous solution of madronin at 15 pounds pressure for thirty minutes did not cause loss of activity.

Surface Activity.—A solution of madronin (0.2%) dissolved in distilled water lowered the surface tension of the water 10 dynes. A one per cent solution did not hemolyze red blood cells.

BIOLOGICAL CHARACTERISTICS

Antibacterial Spectrum.—The antibacterial action of madronin was determined against pathogenic and saprophitic microorganisms by the serial dilution method previously described. Table II shows the degree of activity reported as μg. of madronin per ml. of medium required to inhibit completely the growth of the respective microorganisms after twenty-hour incubation at 37°, unless the conditions are otherwise specified. No antibacterial activity is designated as "no inhibition."

The inoculum dilution for the serial dilution tests in Table II varied slightly, as indicated. This was due to the fact that only the minimum amount of inoculum was used which would give moderately heavy growth in the control tube during the required time of incubation.

In a few cases when the filter paper-diffusion plate method demonstrated that there was absolutely no inhibition, the serial dilution method was not tried. In these instances the diffusion plate inoculum was 1 ml. of a twenty-four hour broth culture of the respective microorganism added to the melted agar, and the length of incubation was also twenty hours.

Inactivating Factors.—While determining the antibacterial spectrum of madronin, it was found that there were various factors in the media employed which somewhat altered the results and made the proper choice of media quite important.

When 10% nonhemolyzed (yellow), sterile beef or horse serum was incorporated into the *M. pyogenes* var. *albus* seeded agar diffusion plate, there was no inactivation of antibacterial activity, but when 10% hemolyzed (red), sterile beef or horse serum was used, there was practically no antibacterial activity.

When 5% sterile, nonhemolyzed beef or horse serum was added to each of the tubes in the serial dilution method, partial inactivation resulted. The amount of this inactivation was found to be dependent upon the serum concentration. Since 3% serum produced only partial inactivation, this amount was added to the media for the assays on microorganisms which required a serum supplement for normal growth.

Brain-heart infusion broth and trypticase-soy-yeast extract broth completely inactivated madronin.

There was no inactivation of madronin when 0.01% cysteine was added to the serial dilution assay tubes or when 0.1% cysteine was incorporated in the agar diffusion plates.

Toxicity.—The LD_{50} for intraperitoneal injection of mice is 3.5 to 4.0 mg. of madronin per 20-Gm. mouse, calculated as 175–200 mg./Kg. for mice. There were no deaths before thirty-six hours.

Four mice (each 20 Gm.) received daily intraperitoneal injections of 1 mg. of madronin in 0.1 ml. of distilled water for sixteen consecutive days apparently without any outward ill effects, as no symptoms of toxicity developed.

The active principle was absorbed from the peritoneum of the mouse and excreted in the urine. Urine was collected by natural voiding on filter papers which were then air-dried; disks were punched out and plated on *M. pyogenes* var. *albus* seeded agar plates. Normal mouse urine and urine collected thirty minutes after the injection of 3 mg. of madronin both showed no antibacterial activity, whereas the urine collected four hours after the injection showed a marked zone of inhibition (15 mm.) of bacterial growth which indicated the presence of an active principle.

DISCUSSION

Madronin is a stable, water-soluble, neutral, yellow solid which is isolated from the leaves of the Madrona tree. It is apparently composed of carbon, hydrogen, and oxygen and contains some water of hydration.

Adsorption and partition chromatography as well as electrophoretic studies (to be reported later) indicate that several chemical entities having similar chemical and antibacterial activities are present in the plant.

Although the substances have not been isolated in pure form, some chemical characterizations have been made. The compounds are reducing agents and appear not to contain carbohydrate or alkoxyl groups. A strong ultraviolet absorption peak is present at 275 mμ which shifts reversibly to a longer wavelength in alkaline solutions.

Despite the fact that it has not yet been obtained in chemically pure form, madronin has an antibacterial activity as great as any substance previously derived from a plant source. Of particular interest is the marked inhibition of the Gram-negative genera *Klebsiella*, *Shigella*, and *Alkaligenes*, despite the fact that closely related bacteria such as the *Escherichia* and *Salmonella* are completely unaffected. The antibacterial spectrum of madronin does not correspond to the pattern of any known antibiotic previously reported in the literature.

SUMMARY

A method is presented for the extraction and concentration of a water-soluble antibacterial principle from the fresh leaves of *Arbutus menziesii*, family *Ericaceae*.

The principle, named madronin, is mainly active *in vitro* against the Gram-positive and acid-fast bacteria and shows very little activity against Gram-negative bacteria except the genera *Klebsiella*, *Shigella*, and *Alkaligenes*.

It is relatively nontoxic to mice and is excreted in an active form in the urine.

The active principle is not inactivated by cysteine or serum *in vitro*.

Physical, chemical, and biological characteristics are described.

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Some New Nitro Amines and Diamines*

By E. B. HODGE

By the reaction of 2-nitro-2-methyl-1-propanol with some aryl aralkylamines, a series of new nitro amines was prepared. Diamines were prepared from these nitro amines by reduction and reductive methylation. Some of the ditertiary amines, prepared by reductive methylation, showed pronounced antihistaminic activity.

JOHNSON (1) has reported the preparation of nitro amines by the reaction of aromatic amines with a nitroparaffin and formaldehyde or with the corresponding nitro alcohol. In these preparations, strongly basic catalysts were used.

It seemed possible that if this reaction could be extended to amines of the type $ArN(CH_2Ar)H$, where Ar represents an aryl group, the resulting nitro amines should be capable of reductive methylation to give compounds which by analogy with other known compounds might be expected to exhibit antihistaminic activity (2). The reactions are illustrated below:

$$ArN(CH_2Ar)H + HOCH_2C(CH_3)_2 \rightarrow ArN(CH_2Ar)CH_2C(CH_3)_2 + H_2O$$

$$\begin{matrix} & & NO_2 & & NO_2 \\ & & Ni & & (I) \end{matrix}$$

$$(I) + 6H_2 + 2CH_2O \rightarrow ArN(CH_2Ar)CH_2C(CH_3)_2 + 4H_2O$$

$$N(CH_3)_2$$

$$(II)$$

When reactions of the type illustrated were tried, it was found that the amines did not react as readily as the more simple aromatic amines; but under proper conditions, fairly good yields of nitro amines were obtained. Reduction and reductive methylation of these products presented no particular difficulties. The nitro amines prepared are listed in Table I. All of these compounds except No. 6 are yellow, but the shade varies from almost orange to an "off white," depending on the substituents on the aryl groups.

The amines listed in Table II were prepared by reduction and reductive methylation of nitro amines prepared as described above.

The antihistaminic activities of these compounds, relative to diphenylhydramine hydrochloride, were determined by Dr. J. N. Spencer, of the Pharmaceutical Research Division, Commercial Solvents Corp., by a method similar to that described by Rocha e Silva and Beraldo (3). They are recorded in the table as per cent activity. Thus, 100 means that the antihistamine activity of the compound was equal to that of diphenhydramine hydrochloride.

EXPERIMENTAL

Nitro Amines.—The most convenient method found for preparing the nitro amines consisted in refluxing a solution of the amine and 2-nitro-2-methyl-1-propanol in *n*-butyl alcohol with a small amount of a mildly basic catalyst such as sodium carbonate or sodium acetate. Under these conditions, most of the reactions were complete in six to twelve hours, although a few took longer. The course of the reaction was followed by separating and measuring the water formed. The nitro amines generally crystallized from the *n*-butyl alcohol on cooling. The preparation of N-benzyl-N-phenyl-2-nitroisobutylamine is given as an example.

A mixture of 18.3 Gm. (0.1 mole) of N-benzylaniline, 11.9 Gm. (0.1 mole) of 2-nitro-2-methyl-1-propanol, 0.3 Gm. of sodium carbonate, and 75 ml. of *n*-butyl alcohol was refluxed for seventeen hours with a trap attached for removing the water. Then the reaction mixture was decanted, cooled, and filtered, yielding 14.8 Gm. of yellow crystals, m. p. 80–83°. Repetition of this process, using the filtrate as the reaction medium, gave 19.3 Gm. of product. Thus, the over-all yield was 60%. The product was crystallized from methanol.

Primary, Tertiary Amines.—Reduction of the nitro amines was carried out by hydrogen in the presence of Raney nickel. An example is given below.

A solution of 29.8 Gm. (0.1 mole) of N,N-dibenzyl-2-nitroisobutylamine in 500 ml. of methanol was reduced in the presence of 10 Gm. of Raney nickel at 1000 p. s. i. and 50°. After reduction, the mixture was concentrated on the steam bath, dissolved in ether, and made just acidic with concentrated hydrochloric acid. A white precipitate formed which was filtered, washed with ether, and dried, yielding 25.5 Gm. of product. Recrystallization from water gave 18 Gm. The product contained

* Received July 17, 1952, from the Pharmaceutical Research Division, Commercial Solvents Corp., Terre Haute, Ind.

TABLE I.—NITRO AMINES OF THE FORMULA $ArCH_2N(Ar')CH_2C(NO_2)(CH_3)_2$

No.	Ar	Ar'	M. P.,[a] °C.	Yield, %	Analyses, % N, Calcd.	N, Found	Cl, Calcd.	Cl, Found
1	C_6H_5	C_6H_5	83.5–84.5	60	9.85	9.61	...	...
2	p-ClC_6H_4	C_6H_5	93–94	50	8.79	8.53	11.12	11.08
3	C_6H_5	p-$CH_3C_6H_4$	109.5–110	69	9.36	9.22	...	...
4	p-$CH_3OC_6H_4$	C_6H_5	101.5–102.5	58	8.91	8.79	...	...
5	C_6H_5	p-$CH_3OC_6H_4$	74.5–76	63	8.91	8.73	...	...
6	C_6H_5	$C_6H_5CH_2$	46.5–47.5	53	9.39	9.15	...	...
7	p-$CH_3OC_6H_4$	p-$CH_3OC_6H_4$	77–78	68	8.15	7.91	...	...
8	p-$CH_3OC_6H_4$	p-$CH_3C_6H_4$	117.5–118	56	8.53	8.45	...	...
9	3,4-$CH_2O_2C_6H_3$	C_6H_5	86–87	51	8.53	8.45	...	...
10	p-$CH_3OC_6H_4$	p-ClC_6H_4	110.5–112	48	8.03	7.93	10.17	10.06
11	C_6H_5	p-$C_6H_5C_6H_4$	120.5–122	50	7.77	7.57	...	...
12	C_6H_5	α-Naphthyl	132–134.5	61	8.38	8.29	...	...

[a] Corrected.

TABLE II.—DIAMINE HYDROCHLORIDES OF THE FORMULA $ArCH_2N(Ar')CH_2C(CH_3)_2.HCl$ (NR₂)

No.	Ar	Ar'	R	M. P.,[b] °C.	Yield, %	Analyses, % N, Calcd.	N, Found	Cl, Calcd.	Cl, Found	Antihistaminic Activity, %[c]
1	p-$CH_3OC_6H_4$	C_6H_5	H	172–174	89[d]	8.73	8.72	11.05	11.14	1.0
2	C_6H_5	$C_6H_5CH_2$	H	149–153 (dec.)	79	9.19	9.01	11.63	11.61	2.0
3	p-$CH_3OC_6H_4$	p-$CH_3C_6H_4$	H	87.5–89[e]	..	8.37	8.24	10.59	10.51	<1.0
4	p-$CH_3OC_6H_4$	p-ClC_6H_4	H	101–104[e]	51	7.89	7.85	10.96	19.67	1.0
5	p-$CH_3OC_6H_4$	α-Naphthyl	H	178–181	33[d]	7.55	7.37	9.56	9.49	...[a]
6	C_6H_5	p-$C_6H_5C_6H_4$	H	221–225.5	..	7.64	7.45	...	...	...[a]
7	C_6H_5	C_6H_5	CH_3	127.5–128.5	73[d]	8.78	8.63	11.12	11.17	100
8	p-ClC_6H_4	C_6H_5	CH_3	187.5–190	55	7.93	7.58	20.07	19.57	100
9	p-$CH_3OC_6H_4$	C_6H_5	CH_3	165–166	46	8.03	7.85	10.16	10.33	50
10	p-$CH_3OC_6H_4$	p-$CH_3C_6H_4$	CH_3	196–197	74	7.72	7.54	9.77	9.86	2
11	p-$CH_3OC_6H_4$	p-$CH_3OC_6H_4$	CH_3	169.5–172	54	7.39	7.14	9.36	9.48	1
12	C_6H_5	$C_6H_5CH_2$	CH_3	104–106	88[d]	8.42	8.25	10.65	10.38	2
13	3,4-$CH_2O_2C_6H_3$	C_6H_5	CH_3	189–191	64	7.72	7.54	9.77	9.69	10
14	p-$CH_3OC_6H_4$	p-ClC_6H_4	CH_3	201–202	70	7.31	7.22	18.50	17.93	10
15	C_6H_5	p-$C_6H_5C_6H_4$	CH_3	175–179	60[d]	7.09	7.02	8.98	8.79	...[a]

[a] Too insoluble to assay.
[b] Corrected.
[c] Of monohydrate.
[d] Of free base; other yields are for hydrochlorides.
[e] Relative to diphenhydramine hydrochloride.

water of crystallization which was removed by drying at 105° at about 1 mm. of mercury.

Tertiary, Tertiary Amines.—Reductive methylations of the nitro amines were carried out by two-step reductions, with the second step in the presence of formaldehyde. An illustrative example is given below.

To a quantity of 31.4 Gm. (0.1 mole) of N-*p*-methoxybenzyl-N-phenyl-2-nitroisobutylamine in 500 ml. of methanol was added 10 Gm. of Raney nickel and the mixture was reduced at 1000 p. s. i. and 50°. Then the bomb was cooled, 25 Gm. of aqueous 37% formaldehyde solution was added, and the reduction was continued under the same conditions as before. After hydrogen absorption had ceased, the catalyst was filtered out and the filtrate was concentrated to give 30 Gm. of residue. This was dissolved in 100 ml. of ether; 25 ml. of ethanol was added, and the solution was made just acid with concentrated hydrochloric acid. The mixture crystallized and was filtered to give 16 Gm.; m. p. 162–165°. Recrystallization was carried out using an ethanol-benzene mixture.

SUMMARY

1. A series of new nitro amines has been prepared by the reaction between 2-nitro-2-methyl-1-propanol and some amines of the type $ArN(CH_2Ar)H$ where Ar represents an aryl group.

2. Reduction of some of the nitro amines led to a series of amines which showed weak antihistaminic activity.

3. Reductive methylation of some of the nitro amines led to a series of amines, some of which showed moderate to strong antihistaminic activity.

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Notes

A Note on the Antagonistic Effect of Histamine Toward the Convulsive Effect of Antihistamines*

By JOSEPH W. E. HARRISSON, DAVID C. SCHECTER, CLARA M. AMBRUS, and JULIAN L. AMBRUS

WYNGAARDEN and Seeners (1) have reviewed recently the toxic effects of antihistamines. In acute intoxication (particularly in children), convulsions occur frequently. Gruhzit and Fisken (2), Wyngaarden and Seeners (1), and Way and Herbert (3) attempted to inhibit this latter effect with barbiturates. During previous studies, we have observed that if guinea pigs received convulsive doses of various antihistamines, the convulsions immediately subsided if the animals were placed in an aerosol apparatus (4) which vaporized a 1% histamine diphosphate solution under 110 mm. Hg pressure. The present study was undertaken to obtain quantitative data on the antagonistic effect of histamine on antihistamine-induced convulsions in mice. Mice present a special problem of antihistamine-histamine antagonism. Bovet and Waltheret (5), Halpern and Ducrot (6), and Mayer and Brousseau (7) have shown that antihistamines potentiate the toxic effect of histamine in mice. We have found (8) that from the point of view of body temperature-decreasing effect, antagonism exists between histamine and antihistamines at certain optimal dose levels. Above and below these dose levels, synergism is evidenced.

MATERIALS AND METHODS

Swiss female mice of 25–30 Gm. body weight from our own colony were used. They were maintained on Rockland complete rat pellets and water *ad libitum* in air-conditioned quarters at 21°. Histamine diphosphate and Neoantergan hydrochloride[1] were dissolved in normal saline. All doses refer to the base. In most experiments an approximate convulsive dose of 45 mg./Kg. of Neoantergan (approximately CD_{90}) was given intraperitoneally. Immediately after the first few convulsions, the desired dose of histamine was given intravenously. The animals were then observed for convulsions during a period of about six hours and for delayed fatal effects for forty-eight hours.

RESULTS AND DISCUSSION

Results are set forth in Table I. It appears that 45 mg./Kg. of Neoantergan caused convulsions in 66 of 74 animals. These convulsions could be decreased by 26–35 mg./Kg. of histamine and abolished by 52–105 mg./Kg. of histamine. Although higher doses of histamine decreased the convulsive effect of Neoantergan, the animals ultimately died, probably because of a synergism between the toxic effect of histamine and Neoantergan. The synergism and

TABLE I

No. Mice Used	Equiv. Dose Neoantergan Base, Mg./Kg.	No. Mice in Convulsions Before Histamine Administration	No. Deaths	Equiv. Dose Histamine Base, Mg./Kg.	No. Mice in Convulsions After Histamine Administration	No. Deaths
6	90	6	2	...	...	...
6	62.5	6	2	...	...	...
6	45	6	0	...	...	...
6	45	...[a]	...[a]	35	0	0
6	45	6	...	17.5	6	2
9	45	6	...	26.25	6[b]	1
7	45	6	...	35	6[b]	0
8	45	6	...	52.5	0	0
6	45	6	...	87.5	0	0
8	45	6	...	105	0	0
6	45	6	...	140	1[b]	0
6	45	6	...	175	0	3[c]
6	45	6	...	210	0	3[c]
6	45	6	...	245	0	3[c]

[a] Histamine was injected intraperitoneally immediately after Neoantergan, i. e., before cramps developed.
[b] Convulsions of mild degree only.
[c] Survivors were moribund and eventually died within forty-eight hours.

* Received April 5, 1952, from the LaWall Memorial Laboratory of Pharmacology and Biochemistry, Department of Pharmacology, Philadelphia College of Pharmacy and Science, Philadelphia.

[1] We are greatly indebted to Dr. R. Paul (Rhone Poulenc Co., Paris) for generously supplying us with this material.

antagonism between toxic doses of histamines and antihistamines in this strain of mice has been reported by us elsewhere (8). If 35 mg./Kg. of histamine was injected intraperitoneally immediately after the Neoantergan injection and before convulsions occurred, this dose was sufficient to prevent the development of convulsions. It thus appears that histamine is able to antagonize the convulsive effects of Neoantergan; however, if high doses are given, the animals die within six to forty-eight hours.

SUMMARY

1. Certain dose levels of histamine are able to antagonize the convulsive effect of Neoantergan in mice.

2. Higher doses of histamine, although they antagonized the convulsive effect of Neoantergan, were followed by the death of the animals.

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A Note on an Improved Method of Analgetic Evaluation*

By WILLIAM B. BASS and MILTON J. VANDER BROOK†

THE ideal method for evaluating analgesia should satisfy several requirements, which have been enumerated by various authors (1–4) and are well known. While numerous techniques have been devised, using both man and animals as test objects (4), no existing method satisfies all of the requirements of the ideal. A procedure which has gained rather wide acceptance for use in animals is that of D'Amour and Smith (5) which was adapted for use with rats from the method of Hardy, Wolff, and Goodell (6), which employs human subjects.

Both of these techniques make use of thermal stimulation; in the D'Amour-Smith method, the response time is measured with a stop watch. This timing procedure introduces two distinct disadvantages. First, as pointed out by Winter and Flataker (7), the reaction time of the operator is included in the measured response time of the rat; when response times are of the order of two or three seconds, this error becomes of considerable importance. Second, the use of radiant heat stimulation, as ordinarily employed, produces considerable eye fatigue in the operator (8).

The modification of the D'Amour-Smith apparatus in use in this laboratory overcomes both of these difficulties by automatic measurement of response times. This is accomplished by the inclusion of an electric stop watch in the circuit of the radiant heat source, plus a circuit-breaking relay which is activated by a photocell. The apparatus is diagrammed in Fig. 1. The photocell is located beneath a hole in the grooved metal tail support. In use, this hole is covered by the tip of the rat's tail. Closing the lamp circuit simultaneously starts the stop watch and turns on the lamp; movement of the tail exposes the photocell to the lamp rays, activating the relay and opening the lamp-stop watch circuit. Thus the rat itself terminates the stimulus and times its duration, lending greater objectivity to the response time measurements. This procedure also eliminates the necessity for the operator to focus steadily on the area of stimulation where the concentrated lamp rays are directed; eye strain is thus avoided.

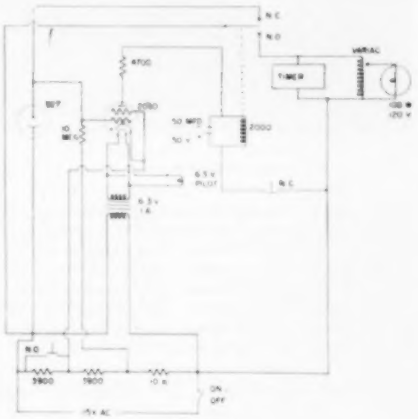

Figure 1.

Other than the modifications mentioned, the apparatus as used here is similar to that described by the originators of the method (5), and is shown in Fig. 2. By means of a photo-flash reflector, the rays from a 100-watt projection lamp are focused on the rat's tail, supported in a grooved metal sup-

* Received April 30, 1952, from the Department of Pharmacology, the Upjohn Company, Kalamazoo, Mich.
† Acknowledgment is made to Clayton D. Alway of the Department of Physics of the Upjohn Company for construction of the apparatus described herein.

TABLE I.—RESPONSE TIMES FOLLOWING I. V. ANALGETICS

Compound	Dose, Mg./Kg.	No. Rats	Average Response Time, Sec. 20 Min. after Injection	40 Min. after Injection	60 Min. after Injection
Uninjected controls	. . .	10	3.6	3.5	4.0
Saline controls	(3.33 cc./Kg.)	6	4.0	4.0	4.1
Metapon HCl	2	6	10	10	9.1
	1	6	9.1	8.2	6.3
Methadon HCl	2	6	9.9	8.4	7.2
	1	6	6.9	5.5	4.5
Morphine SO_4	10	6	9.7	10	10
	5	6	8.6	9.2	7.0
	2	6	6.5	5.3	4.1
Dicodid bitartrate	5	6	10	8.8	7.2
	2.5	6	7.5	5.2	5.0
Demerol HCl	15	6	8.9	7.9	6.2
	10	10	6.9	4.9	4.7
	5	6	4.7	4.3	4.1
	2	10	3.9	4.0	3.9
Codeine phosphate	15	6	7.3	6.6	6.0
	10	6	5.0	4.5	4.2
Dionin HCl	15	6	5.0	4.4	4.4
	10	6	4.5	4.3	4.1

Figure 2.

port. Light intensity is controlled by means of a variable transformer, and voltage across the lamp filament is metered. Voltage is adjusted so that the majority of animals respond in three to five seconds; those not giving three consecutive responses within this interval are discarded. Cutoff time is ordinarily ten seconds, or approximately two and one-half times the average response time.

Tails of the individually caged animals are washed in tepid water and dried. The animal under test is wrapped loosely in a towel and placed in a restraining box with the tail protruding through a slot. After three preinjection readings at ten-minute intervals, the drugs are administered by slow intravenous injection in a volume of 3.33 cc./Kg.

Drugs are dissolved in saline and given as unknowns; sequence of administration is randomized and saline controls are included. Response times are again determined at twenty, forty, and sixty minutes after injection, and averaged for each group. Uninjected control rats show a standard deviation of ±0.4 second, or about 9% of the average of 4.4 seconds; this is comparable to results of others (1, 5) using the unmodified D'Amour-Smith apparatus.

Using the technique as described above, the analgetics metapon, methadon, morphine, Dicodid, Demerol, codeine, and dionin were arranged in that order of decreasing activity. This is in fair agreement with clinical experience. Results are summarized in Table I.

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Book Reviews

Biochemical Preparations. Vol. 2. ERIC G. BALL, Editor-in-Chief. John Wiley and Sons, Inc., 1952. vii + 109 pp. 15.5 x 24 cm. Price $3.00.

While the title of this book (the second in a contemplated continuing series) implies a restricted biochemical interest, the preparations and chemicals described possess considerable pharmaceutical interest. In general, each monograph includes a clear and concise statement of the principle involved, the starting material required, the procedure to be followed, properties and criteria of purity of the finished product, and other methods of preparation. Methods of preparation which are adequate are described for the following products: Cytochrome C; Cucurbit Seed Globulin; Oxycaseia; Phosvitin, Sodium Salt; Crystalline Lactate Dehydrogenase; Sodium Pyruvate; Phosphoryl-enolpyruvic Acid; 1-α-Glycerophosphoric Acid; Glucose-6-Phosphate; C^{14} Uniformly Labeled Sucrose; Fructose-1, 6-Diphosphate; *myo-* and DL-*epi*-Inosose-2; Inositol Monophosphate; DL-Glutamic Acid Monohydrate; L-Aspartic Acid; S-Benzyl-1-Cysteinylglycine; α-Methyl Carbobenzoxy-L-Glutamate; N-Carbobenzoxy-γ-1-Glutamyl-S-Benzyl-1-Cystinylglycine; Glutathione; Reduced Diphosphopyridine Nucleotide; Phosphorylcholine; Oleic Acid and Methyl Oleate; and Methyl Ricinoleate.

Acids and Bases. Their Quantitative Behaviour. By R. P. BELL. John Wiley and Sons, Inc., New York, 1952. vi + 90 pp. 17 x 11 cm. Price $1.50.

The aim of the author of this little book is to present a brief and unified account of the applications of acids and bases through a wide variety of chemical problems. The first two chapters, elementary in nature, cover material on the nature of acids and bases and acid-base equilibria in water which is readily available from other sources. The behavior of acids and bases in nonaqueous solvents is discussed in chapter 3 and interionic attraction in acid-base equilibria in chapter 4. Chapter 5 discusses briefly and clearly the correlation of acid-base strengths with molecular structure, and the catalytic power of acids and bases in many reactions is covered in the sixth chapter. General references to books or review articles are listed at the end of each chapter. The book is provided with an adequate index, and its publication represents an interesting addition to *Methuen's Monographs on Chemical Subjects.*

Review of Pharmacy. 7th ed. By GEORGE W. FIERO. John Wiley & Sons, Inc., New York, 1952. 122 pp. 16 x 23.5 cm. Price $3.25.

The object of this book is to furnish condensed information relating to drugs in the latest editions of *The United States Pharmacopeia* and *The National Formulary.* It may be used to advantage by pharmacy students in preparing for examinations and by the pharmacist as a reference book. It is doubtful, however, that the book can possess the significance or serve the purpose that it might have in 1927 when the first edition was published.

Symposium on Radiobiology. The Basic Aspects of Radiation Effects on Living Systems. Ed. by JAMES J. NICKSON. John Wiley & Sons, Inc., New York, 1952. xii + 465 pp. 15.5 x 23.5 cm. Price $7.50.

A Symposium on Radiobiology was held at Oberlin College in June, 1950, in an attempt to present a résumé of the state of knowledge of the field at that time. This book reports the 23 papers presented to the Symposium, covering four separate phases of radiobiology. These four separate phases include (1) a survey of the physical interaction of ionizing radiation and matter; (2) an explanation of the chemical changes arising from the transfer of physical energy; (3) an examination of the biochemical effects; and (4) a discussion of the changes occurring in living tissue. The authors have attempted to give full development to each subject from its basic aspects to the most complex phases.

A Guide to the History of Science. By GEORGE SARTON. Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York; 1952. xviii + 316 pp. 16 × 23.5 cm. Price $7.50.

This book is concerned with all branches of the natural and exact sciences. It is divided into two main parts, the first and shorter of which is based upon lectures explaining the purpose and meaning of the history of science which were delivered by the author in several European universities. The second and much longer part of the book presents a bibliographical summary prepared for the guidance of scholars interested in any phase of the history of science, which can be used to advantage by historians of science. The author, who is editor of *Isis and Osiris* and a professor in Harvard University, clearly stresses the fact that historians of science must know both science and history, and that knowledge of one is insufficient without an understanding of the other. The bibliographies on chemistry, medicine, and pharmacy and the complete list of periodicals devoted partially or entirely to the history of science should appeal particularly strongly to the student of the history of pharmacy.

Skin Therapeutics. Prescription and Preparation. By M. K. POLANO. Elsevier Publishing Co., Houston, Texas, 1952. xiv + 276 pp. 16 x 23.5 cm. Price $6.50.

The author of this book is Head of the Dermatological Department, Municipal Hospital, The Hague, Netherlands. The book is of pharmaceutical interest because one of its aims throughout has been to stress the importance of the art of prescribing by dermatologists. In addition, it contains many

formulas and directions for their preparation which should assist pharmacists in compounding problems frequently met with in connection with ointments, lotions, and other dermatological preparations. The author has attempted to include information on both old and new topical therapeutic agents and to review nearly all types of ointment bases and lotion vehicles. During recent years many publications have appeared concerning new types of ointment bases and lotion vehicles which are not adequately treated in this book. This, however, does not detract from its usefulness both to the pharmacist and to the dermatologist.

A particularly useful feature is the number of tables comparing dermatological medicaments of the pharmacopeias and formularies of Belgium, Denmark, France, Germany, Great Britain, Italy, The Netherlands, Spain, Sweden, and the United States. These tables are of especial value to pharmacists who receive prescriptions written in foreign countries, because they give the equivalents of many drugs otherwise difficult to determine.

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BOOKS RECEIVED

Glycols. Edited by GEORGE O. CURME, JR., and FRANKLIN JOHNSTON. Reinhold Publishing Corp., New York, 1952. xii + 389 pp. 16 x 23.5 cm. Price $12.00.

A Guide to the History of Science. By GEORGE SARTON. Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York; 1952. xviii + 316 pp. 16 x 23.5 cm. Price $7.50.

Dynamic Aspects of Biochemistry. By ERNEST BALDWIN. 2nd ed. Cambridge University Press, New York, 1952. xx + 544 pp. 14 x 22 cm. Price $5.00.

A Laboratory Manual for Inorganic Pharmaceutical Products. By ROBERT F. DOERGE and CHARLES O. WILSON. Hemphill's, Austin, Texas, 1952. 196 pp. 23 x 27.5 cm.

Nuttall's Travels into the Old Northwest: An Unpublished 1810 Diary. By JEANNETTE E. GRAUSTEIN. No. 1/2 of Volume 14 of Chronica Botanica. Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York; 1952. Illustrated. viii + 88 pp. 17.5 x 26 cm. Price $3.00.

International Health Organizations and Their Work. By NEVILLE M. GOODMAN. The Blakiston Co., Philadelphia, 1952. xi + 327 pp. 16 x 23.5 cm. Illustrated. Price $6.50.

Methods and Reactions of Organic Analysis. Vol. I. General Methods of Analysis. By MAURICE PESEQ and PIERRE POIRIER. Masson & Cie, Paris. 276 pp. 17 x 25.5 cm. Price 1,800 fr.

A Study Manual for Inorganic Pharmaceutical Products. By TONY E. JONES and CHARLES O. WILSON. Hemphill's, Austin, Texas, 1952. 144 pp. 23 x 27.5 cm.

Acids and Bases. Their Quantitative Behaviour. By R. P. BELL. John Wiley & Sons, Inc., New York, 1952. vi + 90 pp. 17 x 11 cm. Price $1.50.

Ultraviolet Radiation. By LEWIS R. KOLLER. John Wiley & Sons, New York, 1952. ix + 270 pp. 15.5 x 24 cm. Illustrated. Price $6.50.

Useful Drugs. 15th ed. J. B. Lippincott Co., Philadelphia, 1952. xi + 262 pp. 19.5 x 13 cm. Price $2.50.

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Announcement

AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE

The 119th meeting of the American Association for the Advancement of Science and participating affiliated and associated societies will be held in St. Louis, December 26–31, 1952.

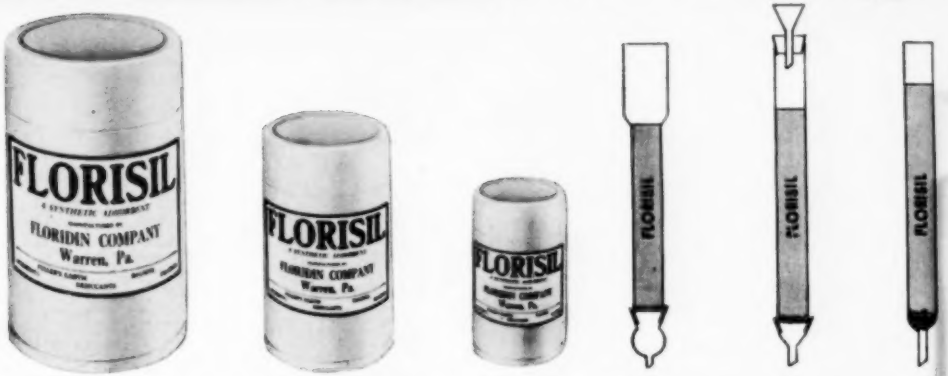
The program for the Pharmacy Subsection of the Medical Sciences is now being made up. At least four sessions will be held.

Individuals connected with the research and project development departments of pharmaceutical laboratories, accredited schools of pharmacy, pharmacy associations, and practicing pharmacists are especially invited to contribute scientific papers for these sessions.

Authors who wish to present papers before the Subsection are requested to send in titles of papers as soon as possible. All titles for inclusion in the printed program must be in by November 1.

Send titles to: George F. Archambault, *Chairman*, Subsection Np, AAAS, Division of Hospitals, U. S. Public Health Service, Washington 25, D. C.

'n the Laboratory ..in the Plant



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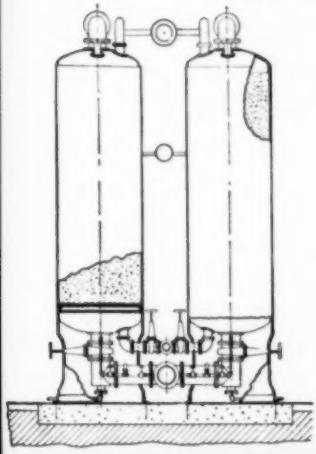

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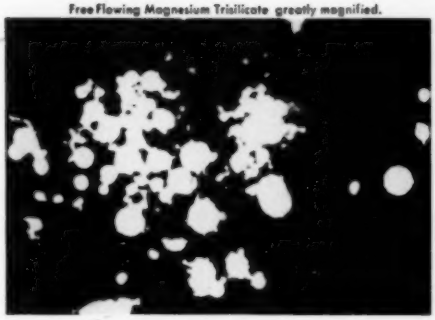

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